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ETHANOL-INDUCED REGULATION OF THE HUMAN DOPAMINE TRANSPORTER

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by

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Dedication

This dissertation is dedicated to my husband, Joey Methner, my parents, John and Jan Riherd, and my siblings Dustin and Morgan Riherd

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ETHANOL-INDUCED REGULATION OF THE HUMAN DOPAMINE TRANSPORTER

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The University of Texas at Austin, 2009

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The dopamine transporter (DAT) is a plasma membrane-bound protein, localized on peri-synaptic terminals of dopaminergic (DA) neurons. DAT is responsible for terminating DA signaling by rapid removal of the transmitter from the synaptic cleft region. DA signaling relies on a critical balance between release and removal of the neurotransmitter within synaptic clefts. Recycling of DAT between intracellular endosomal compartments and the plasma membrane regulates DAT function. This dynamic trafficking occurs in both a constitutive and regulated manner to increase or decrease the number of transporters on the cell surface available for transmitter reuptake. Therapeutic drugs and/or drugs of abuse, including psychostimulants and ethanol, cause maladaptive changes in DA signaling in mesolimbic areas of the brain, leading to addictive behaviors. DAT is the primary site of action for psychostimulants such as, cocaine, methylphenidate, and amphetamine. These drugs can alter the function and/or

regulation of the transporter. Ethanol, one of the most widely abused drugs in society, is known to activate DA pathways in reward and reinforcement areas of the brain. However, the effect of ethanol on DAT function and regulation is less clear. The studies presented here explore the action of ethanol on DAT function in mammalian cell systems, and the subcellular trafficking mechanisms that regulate the transporter. To delineate mechanisms of ethanol action on DAT, several lines of HEK-293 cells stably expressing DAT or ethanol-insensitive DAT mutants were generated. Short-term ethanol exposure was found to potentiate DAT function, and ethanol sensitivity is mediated by specific amino acids in the first intracellular loop. This increase in function was accompanied by an enhancement of DAT expressed on the cell surface. The changes in DAT localization and the absence of consensus phosphorylation sites in the ethanol sensitive regions of the transporter, led to the hypothesis that ethanol modulates DAT uptake by altering the dynamic trafficking of the transporter. In the present studies, we found ethanol directly regulates DAT function by altering specific step of the endosomal recycling pathway. Further analysis of the ethanol-sensitive first intracellular loop revealed this region might also play a role in conformational changes required for substrate binding. The findings presented in these studies describe a novel molecular mechanism of ethanol action on DAT, and provide a framework to further understand the action of ethanol on synaptic dopamine regulation.

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List of Abbreviations

DA	Dopamine
DAT	Dopamine Transporter
GAT1	γ-aminobutyric acid Transporter
NET	Norepinephrine Transporter
SERT	Serotonin Transporter
ADHD	Attention-Deficit / Hyperactivity Disorder
NAc	Nucleus accumbens
SLC6	Solute Carriers 6 gene family
UTR	Untranslated Region
VNTR	Variable Tandem Repeats Polymorphism
SNP	Single Nucleotide Polymorphism
C-terminus	Carboxyl Terminus
N-terminus	Amino Terminus
TM	Transmembrane Region
IL	Intracellular Loop
EL	Extracellular Loop
GLYT1 or 2	Glycine Transporter 1 or 2
ER	Endoplasmic Reticulum
[^{3}H]DA	[^{3}H]Dopamine
G130T	Glycine at position 130 mutated to Threonine

IGLF	Isoleucine at position 137, Glycine at position 130, Leucine at position 138, and Phenylalanine at position 123 mutated to the corresponding NET residues, Phenylalanine, Threonine, Phenylalanine, and Tyrosine, respectively
PICK1	Protein that Interacts with C Kinase
Eps15	Epidermal Growth Factor Pathway Substrate Clone 15
Eps15R	Eps15-related Protein
TfR	Transferrin Receptor
PKC	Protein Kinase C
PKA	Protein Kinase A
PMA	Phorbol 12-Myristate 13-Acetate
Nedd4-2	Neural Precursor Cell Expressed, Developmentally Downregulated 4-2
PI 3-K	Phosphatidylinositol-3-Kinase
MAPK	Mitogen Activated Protein Kinase
MKP3	MAPK Phosphotase
eGFP	Enhanced Green Fluorescent Protein

CHAPTER ONE: INTRODUCTION

Within the central nervous system, chemical neurotransmission allows for rapid communication between neurons and target organs. Once thought to be purely electrical in nature, the concept of chemical communication was originally hypothesized by Thomas Elliot at the turn of the 20th century, and later proven by the Nobel Prize winning work of Otto Loewi in the 1920s (Zigmond 1999). Chemical neurotransmission was finally accepted by the mid 20th century, and soon led to the identification of neurotransmitters that are responsible for the chemical communications. Neurotransmitters are chemicals released from neurons into synaptic clefts that act on specific post-synaptic receptors, which in turn, activates signal transduction cascades. These signals can eventually lead to various biological and behavioral responses of the organism. The discovery of one neurotransmitter, dopamine (DA), by Arvid Carlsson was not without controversy. Thought to be purely a metabolite of another neurotransmitter, norepinephrine, Dr. Carlsson's work in the 1950s-1960s, which eventually lead to a Nobel Prize, proved DA was an important neurotransmitter involved in neural pathways controlling behaviors such as movement, motivation, and reward (Abbott 2007).

During the same time period of Dr. Carlsson's important discoveries about DA, another future Nobel Prize winner, Julius Axelrod, was demonstrating how neurotransmission is regulated. The duration and strength of neurotransmission

is regulated by neurotransmitter transporter proteins, which remove neurotransmitters from the synaptic cleft (Hertting and Axelrod 1961). Axelrod first demonstrated the concept of a reuptake mechanism by his experiments measuring the reuptake of neurotransmitters into sympathetic nerve terminals. Later, specific transporters were identified for several neurotransmitters, including dopamine. Neurotransmitter transporters, such as the dopamine transporter (DAT), play a crucial role in maintaining homeostatic neurotransmission, and are responsible for regulating the precise temporal and spatial aspects of neurotransmission.

DA signaling relies on a critical balance between release and removal of the neurotransmitter within synaptic clefts. Therapeutic drugs and/or drugs of abuse, cause maladaptive changes in DA signaling, leading to addictive behaviors. DAT is the primary site of action for psychostimulants such as, cocaine, methylphenidate, and amphetamine. These drugs can alter the function and/or regulation of the transporter. Psychostimulant-induced modulation of uptake alters synaptic DA levels in the brain, and plays a critical role in mediating the mechanism of action of the drug. Ethanol, one of the most widely abused drugs in society, is known to activate DA pathways in reward and reinforcement areas of the brain. However, the effect of ethanol on DAT function and regulation is less clear. The studies presented here explore the action of ethanol on DAT function, and the subcellular trafficking mechanisms that regulate the transporter.

1.1 Dopamine Transporter

The neurotransmitter, dopamine (DA), plays a crucial role in the neural circuits that drive motor function, emotion, cognitive function, and reward behaviors. Dependent on extracellular concentrations of the transmitter, DA signaling is maintained by a critical balance of DA release and removal from synaptic clefts (Torres et al. 2003b). The duration and strength of DA neurotransmission is regulated by rapid removal of the transmitter via reuptake by DAT (Nelson 1998). DAT is a member of the Na^+/Cl^- family of monoamine transporters including the γ-aminobutyric acid (GAT1), norepinephrine (NET), and serotonin (SERT) transporters (Torres et al. 2003b). Altered DAT function and/or expression has been associated with several neurological disorders including Parkinson's disease, Tourettes's syndrome, attention-deficit / hyperactivity disorder (ADHD), depression, and drug abuse (Seeman and Niznik 1990, Dougherty et al. 1999, Laasonen-Balk et al. 1999, Vandenbergh et al. 2000, Cheon et al. 2004, Nutt et al. 2004, Mehler-Wex et al. 2006, Perona et al. 2008, Zhu and Reith 2008). These findings highlight the importance of DAT in the pathophysiology of several neuropsychiatric disorders, and understanding transporter biology has increasingly significant clinical relevance in the treatment of these diseases.

1.1.1 DAT localization

Within the central nervous system, DAT is expressed in cell bodies, dendrites, axon membranes, and pre-synaptic dopaminergic terminals (Ciliax et al. 1995). There is also a regional heterogeneity of DAT expression within the brain with the highest level of expression reported in basal ganglia areas (Cerruti et al. 1993, Hall et al. 1999). Within the basal ganglia, higher DAT levels have been reported in the nucleus accumbens (NAc) core compared with the shell (Nirenberg et al. 1997a). This differential expression in the NAc underlies some of the functional differences and sensitivity between shell and core in response to psychostimulants (Pontieri et al. 1995, Nirenberg et al. 1997a). In addition to the neuronal localization of the transporters, DAT has also been found in peripheral locations including the stomach, pancreas, and kidney (Eisenhofer 2001).

On a subcellular level, immunogold staining revealed DAT is primarily localized in peri-synaptic areas outside of synaptic active zones (Nirenberg et al. 1996, Hersch et al. 1997). DAT labeling was prominent on the plasma membrane and smooth endoplasmic reticulum of dendrites and dendritic spines of the substantia nigra (SN) and ventral tegmental area (VTA), and along plasma membranes of varicose axonal regions in striatum (Nirenberg et al. 1996, Nirenberg et al. 1997b). The localization of DAT outside the synaptic active zones creates a concentration gradient of DA, drawing the transmitter out of the synaptic region (Nirenberg et al. 1996). Therefore, the specific cellular

localization of the transporter contributes to the termination of dopaminergic neurotransmission.

1.1.2 Molecular characterization of DAT

The gene family of neurotransmitter transporters, solute carriers 6 (SLC6), was first identified in the early 1990s with the cloning of GAT1 and NET (Guastella et al. 1990, Nelson et al. 1990, Pacholczyk et al. 1991). These important discoveries reveal significant amino acid sequence homology between the transporters and were soon followed by the isolation, cloning and characterization of the DAT gene (DAT1; SLC6A3) in rat, bovine, and human brain (Giros et al. 1991, Kilty et al. 1991, Shimada et al. 1991, Usdin et al. 1991).

The human DAT1 gene was mapped to the distal end of chromosome five (5p15.3), and spans approximately 65kb containing fifteen exons (Giros et al. 1992, Vandenbergh et al. 1992). Further analysis of the gene revealed a 40bp region in the 3' untranslated region (UTR) which contains a variable number tandem repeats (VNTR) polymorphism ranging from three to eleven copies (Vandenbergh et al. 1992). Differences in the allelic frequency of this polymorphism can vary between global populations and ethnic groups, suggesting a potential link between genetic variations of the transporter and the the vulneribility of specific human populations to various neuropsychiatric disorders (Mitchell et al. 2000).

Association studies have implicated the 3'UTR VNTR polymorphism, and other single nucleotide polymorphisms (SNPs) with neuropsychiatric disorders in various human populations (Ueno 2003, Haddley et al. 2008). For example, the polymorphism in the 3'UTR may confer vulnerability to alcoholism (Ueno et al. 1999, Kohnke et al. 2005, Lind et al. 2009, Vaske et al. 2009) and/or contribute to the severity of withdrawal symptoms in alcoholics (Sander et al. 1997, Schmidt et al. 1998, Le Strat et al. 2008). Additionally, SNPs in exon 2, intron 4, and in the 3' UTR of the DAT1 gene have also been associated with alcohol consumption behaviors and withdrawal (Le Strat et al. 2008, Lind et al. 2009). However, several groups have found no relationship between alcohol use and the DAT1 gene 3'UTR VNTR polymorphism (Parsian and Zhang 1997, Franke et al. 1999, Foley et al. 2004). The lack of consensus on the association of genetic polymorphisms with alcohol-related behaviors could be attributed to differences in populations and/or severity of disease between groups tested.

While the polymorphic variants of the DAT1 gene may be important in conferring vulnerability to specific neuropsychiatric diseases, little is known of its transcriptional and translational regulation. Since the 3' VNTR polymorphism described above is in an untranslated region of the gene, several groups have suggested it may serve as a *cis*- or *trans*-acting transcriptional regulatory element (Heinz et al. 2000, Jacobsen et al. 2000, Mill et al. 2002, Fuke et al. 2005). Analysis of the 5' region of the DAT1 gene suggests the presence of strong, non-specific promoter activity (Kouzmenko et al. 1997, Sacchetti et al.

1999). However, since DAT has a highly restrictive tissue-specific expression indicating there are uncharacterized silencing elements in the distal 5'-flanking region (Sacchetti et al. 1999). Additionally, the nuclear receptor, Nurr1, has been reported as a DAT1 transcription enhancer working through an unidentified response element (Sacchetti et al. 2001). Enhancer elements were also identified in introns 9, 12, and 14. Furthermore, differences in expression of two alleles of intron 14 suggested polymorphic variation in DAT1 gene expression (Greenwood and Kelsoe 2003). Particular combinations of polymorphic variants in regions of DAT1 involved in transcription and translation of the gene could contribute to differences in populations with various neuropsychiatric diseases.

1.2 Structure and Function of DAT

Successful cloning of neurotransmitter transporters in the early 1990s, led to the evaluation and identification of DAT residues and structural motifs that are critical to its function. Proper substrate binding, translocation, and regulation of transporter function are dependent on DAT structure and cell membrane orientation. DAT mediates DA uptake, and the inefficient uptake of norepinephrine (Giros et al. 1992), through a sodium symport mechanism. Drugs of abuse, such as the psychostimulants cocaine and amphetamine target DAT and disrupt DA uptake by interacting with specific amino acids. The identification of critical residues through various experimental and computational methods has advanced our understanding of transporter function and drug action.

1.2.1 DAT topology

Human DAT contains 620 amino acids with an estimated size of 55-90kDa, depending on glycosylation state (Giros et al. 1991). Hydrophobicity analysis of DAT amino acid sequence, and the apparent lack of a signal sequence, revealed monoamine transporters are polytopic membrane proteins containing 12 putative transmembrane (TM) regions with intracellular N- and C-termini (Giros et al. 1991, Kilty et al. 1991, Pacholczyk et al. 1991, Shimada et al. 1991). The transporter also contains a large extracellular loop between TM3 and TM4, which contains several N-linked glycosylation sites (Li et al. 2004). The putative transmembrane domains, and intracellular localization of the termini were initially experimentally confirmed using immunoperoxidase and immungold electron microscopy (Hersch et al. 1997).

An important breakthrough for monoamine transporter biology came in 2005 with the elucidation of the crystal structure of a bacterial homologue (*Aquifex aeolicus*) of Na^+/Cl^- transporters, the leucine transporter ($LeuT_{Aa}$) (Yamashita et al. 2005). Although there is only a 25% homology of sequence between $LeuT_{Aa}$ and DAT, clusters of conserved amino acids are observed throughout the structure, including residues as critical for substrate binding and transporter function (Figure 1.1.A). Furthermore, a recent computational modeling of DAT using the $LeuT_{Aa}$ as a structural template, found the overall topological arrangements, the highly conserved TM regions, and substrate binding domains are almost completely superimposable (Indarte et al. 2008).

Yamashita et al. (2005) described the transporter as a shallow 'shot glass' shape with the opening of the glass facing the extracellular space, lacking a solvent accessible pore that spans the protein. The crystal structure was formed in complex with the leucine substrate and Na^+ ions, and suggest the presence of extracellular and intracellular gates. This finding supports the two gate theory (or 'alternating access model') of transporter uptake dynamics previously purposed (Lester et al. 1996). The twelve TM regions are α-helical, and the core TM regions (TMs 1-10) appear to form a pseudo two-fold axis perpendicular to the membrane involving TMs 1-5 in one "fold" and TMs 6-10 in the second. Specifically the orientation of TMs 1-5 in the membrane are superimposable onto the orientation of TMs 6-10 by rotation of ~180° (Figure 1.1.B). These novel findings could not be recognized with previous analysis of the amino acid sequence, and could have possible implications in understanding of substrate and drug binding of the transporter.

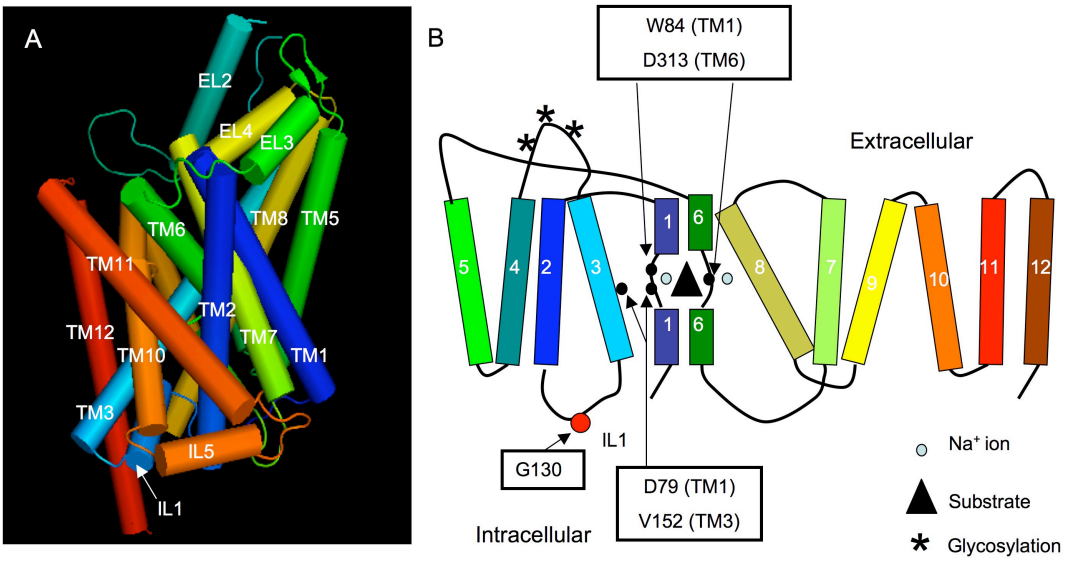

Figure 1.1: DAT structure and topology. A. Structure of a neurotransmitter transporter, based on $LeuT_{Aa}$ crystal structure. **B.** Topological arrangement of DAT. The 'hinge' regions of TM1 and TM6 are the primary areas that make up the putative substrate and Na^+ binding pockets. D79 and V152 are critical amino acids involved in substrate binding. The W84 and D312 residues have been identified as Na^+ binding sites. G130 has also been identified as an ethanol sensitive site. Neurotransmitter transporter structure was generated using MacPyMol 8 software.

1.2.2 Electrophysiological properties of DAT

It has long been accepted that monoamine transporters require an active transport mechanism. The thermodynamically uphill transport of substrate by DAT occurs down an electrochemical gradient, and requires the co-transport of two Na^+ ions and one Cl^- ion (McElvain and Schenk 1992, Chen and Reith 2003). The overall rate of uptake, which was estimated using electrochemical techniques, was found to be 2-5 DA molecules per second per DAT (Povlock and Schenk 1997, Cragg and Rice 2004). At physiological pH, dopamine mostly

exists in the positively charged form (Berfield et al. 1999); therefore the uptake process (DA^+:Na^+:Cl^- = 1:2:1) produces the net import of two positive charges per uptake cycle.

Neurotransmitter transporters have been shown to produce substrate-induced currents (Sonders et al. 1997), capable of membrane depolarization (Carvelli et al. 2004) and a constitutive leak current (Ingram et al. 2002). Electrophysiological studies in midbrain dopamine neurons have revealed DAT may also produce uncoupled Cl^- conductance, which can be blocked by uptake inhibitors. These findings revealed a possible DAT-mediated alternative mechanism to produce neuronal excitability, and regulation of DA release (Ingram et al. 2002).

1.2.3 Structural basis for substrate binding

The identification of specific amino acids and networks of amino acids involved in substrate binding and translocation is critical in understanding the proper function and physiological properties of the transporter. Evidence from chimeras between DAT and NET originally identified regions involved in selective substrate binding, cocaine affinity, translocation and ion dependence (Buck and Amara 1994, Giros et al. 1994, Buck and Amara 1995). DAT and NET are the closest related transporters in the Na^+/Cl^- family of transporters, and have an overall 78% homology of their amino acid sequences (Kilty et al. 1991). While DAT and NET can both remove DA and NE, their pharmacological profiles differ.

The carboxyl end of the transporter (TM9-carboxy-terminal tail) was originally identified as important in determining substrate affinity and steroselectivity for high affinity substrates (such as DA), while the regions between TM6 and TM8 were identified as important in mediating the affinity of cocaine, a competitive inhibitor of DAT (Giros et al. 1994, Buck and Amara 1995). Chimera studies between the slow bovine DAT and human DAT revealed TM3 also was a critical region in facilitating DA uptake and cocaine binding (Lee et al. 1998). However, many chimeras lack functional activity, limiting this approach in identifying specific networks of residues involved in transporter activity.

Site-directed mutagenesis analysis provided additional insights into the structure/function of DAT. In identifying the dopamine-binding site, researchers focused on hydrophobic and charged amino acids, which would bind monoamines, in the highly conserved TM regions. Aspartic acid 79 in TM1 of human DAT, and the corresponding amino acid in NET and SERT, was found to be a site that is presumed to directly interact with monoamines (Figure 1.1.B). Mutation of D79, inhibits binding of substrate without altering cell surface expression of the transporter (Kitayama et al. 1992). Investigation of the TM3 region involvement in substrate binding, found valine 152 (Figure 1.1) in human DAT crucial in interacting with DA (Lee et al. 2000). Tryptophan 84 and aspartic acid 313 in TM1 and TM6, respectively, were found to play a role in Na^+ interaction (Figure 1.1.B). Mutations at W84 and D313 suggested Na^+ interaction with the transporter facilitates substrate binding and translocation (Chen and

Reith 2003). Further site-directed mutation studies have revealed other amino acids that may play a critical, but not clearly defined, role in transporter function.

These earlier site-directed mutational findings are in agreement with the recent computational analysis of DAT based on the $LeuT_{Aa}$ crystal structure (Yamashita et al. 2005, Indarte et al. 2008). In these studies, distinct regions of TM1 and TM6 were found to be the probable dopamine binding sites, and sites of Na^+ interaction. The structures indicate TM1 and TM6 are partially unwound in center of α-helices, forming short hinge-like regions and exposing the main chain carboxyl regions (Figure 1.1.B). This area, along with interactions with TM3 and TM8, form a binding pocket for DA and the two Na^+ ions (one Na^+ at the hinge region of TM1, and the other at the hinge region of TM6). The D79 residue, previously identified as critical in substrate binding (Kitayama et al. 1992), resides in the hinge region of TM1, the area that makes up part of the substrate-binding pocket. The human DAT structure model suggests the side chain of V152 in TM3, also previously identified as a critical residue (Lee et al. 2000), directly interacts with DA in the binding pocket (Indarte et al. 2008). Furthermore, the putative site of Na^+ interaction on central TM1 and TM6 (Figure 1.1.B) are in the regions purposed by Chen and Reith (2003). Valence calculations of the crystal structure yielded sodium-specific valence for these areas, supporting previous mutational analysis.

1.2.4 Structural basis for substrate translocation

In addition to their electrophysiological properties, ion channels and neurotransmitter transporters display gating properties with selective high-affinity binding sites for substrate and ions. However, the gating properties of neurotransmitter transporters, unlike ion channels, appear to contain two putative gates at the intracellular and extracellular faces of the transporter (Lester et al. 1996, Yamashita et al. 2005). Mutational and computational analysis of DAT permeation have identified putative networks of residues which contribute to gating of the transporter and allow for conformational changes to occur during the substrate translocation process.

The outward facing conformation of DAT is stabilized by interactions of an intracellular network of residues. This intracellular gate occludes the substrate and ion binding sits from the cytoplasm, and is composed of interconnection of the N-terminus/TM1 (R60) region with IL3/TM6 (V259, S334, Y335) and TM8 (E428 and D436) region on of the transporter (Loland et al. 2002, Loland et al. 2004, Kniazeff et al. 2008). The R60 forms a putative salt bridge with D436 and a cation-π interaction with Y335. Mutations of these highly conserved residues block DA transport by inhibiting DAT conformational changes during the translocation process (Loland et al. 2004). This network of amino acids likely serve to stabilize the substrate binding pocket formed by TM1 and TM6 and the outward facing conformation (Yamashita et al. 2005, Kniazeff et al. 2008). The less complex extracellular gate, which forms with the inward facing conformation,

is composed of a putative salt bridge between R85 and D476 in TM1 and TM 10, respectively (Yamashita et al. 2005, Huang and Zhan 2007).

Reorganization of DAT structure during the substrate translocation process is initiated by the interaction of Na^+ in the core of the transporter at the unwound and flexible regions of TM1 and TM6 (Chen et al. 1999, Yamashita et al. 2005). Binding of the Na^+ ions likely reorganizes the internal DAT structure, and stabilizes the structure of the substrate-binding pocket, allowing dopamine to bind at TM1 and TM6 (Yamashita et al. 2005, Indarte et al. 2008). Recent modeling of DAT suggested a low-affinity, secondary substrate docking site at the extracellular interface of TMs 1, 6, 10, 11 and EL3 and 4 (Indarte et al. 2008). This area may serve as a molecular 'waiting room' for substrate, and substrate enters the primary binding site with the presence of the ion interactions within the transporter. While the conformational changes that occur after the binding of the substrate have yet to be completely understood, it is thought the flexible unwound regions of TM1 and TM6 act as joints to open and close the two transporter gates (Yamashita et al. 2005). In addition to the highly conserved substrate binding sites and TM region gates, surface exposed areas of the transporter have also been shown to indirectly maintain the open or closed configurations. The intracellular and extracellular ends of the TM2 region bend inwards towards TM1 and TM6 forming a pincer-like configuration. The interaction between the pincer regions and TM1 and TM6 play an indirect role in substrate and cocaine binding (Sen et al. 2005). Furthermore, evidence from the

$LeuT_{Aa}$ crystal structure suggest transporter loops (IL1, EL2, and EL4) shift during substrate translocation and possibly contribute to opening and closing the permeation pathway.

1.2.5 DAT glycosylation

Substrate binding, translocation, and trafficking of DAT can be influenced by post-translational modification. Prior to the cloning of the transporter, researches investigated the molecular characteristics of DAT using photoaffinity binding in rat brain, and found DAT was heavily glycosylated (Grigoriadis et al. 1989, Sallee et al. 1989). Further investigation with deglycosylation enzymes suggested the carbohydrate moiety of the transporter is asparagine-linked (*N*-linked) with high amounts of sialic acid (Lew et al. 1991). DAT contains three putative glycosylation sites on the large EL2 region, N181, N188, and N205 (Figure1.1.B) (Torres et al. 2003a, Li et al. 2004). Comparisons of DAT molecular mass between brain regions, developmental stage, and species revealed a microheterogeneity of transporter glycosylation, suggesting cell type specificity in post-translational processing (Lew et al. 1992, Patel et al. 1994, Vaughan and Kuhar 1996). These differences in glycosylation state alter the functional expression of DAT on the cell surface and may account for differences in DA uptake kinetics between tissues and cell types (Patel et al. 1993, Patel et al. 1994, Povlock and Schenk 1997). Pharmacological deglycosylation or point mutations of the *N*-linked glycosylation sites, decreases surface expression of

the transporter, increases endocytosis of the immature DAT (non-glycosylated transporter), alters inhibitor (including cocaine) sensitivity, and impairs DA uptake (Daniels and Amara 1999, Torres et al. 2003b, Li et al. 2004). However, complete mutation of the three *N*-linked glycosylation sites revealed the small portion of immature DAT expressed on the cell surface is functional (Torres et al. 2003a, Li et al. 2004). These studies suggest glycosylation plays a role in proper transporter trafficking and folding, but is not required for function.

1.2.6 DAT oligomerization

The structural and functional characteristics of DAT outlined above describe the transporter as a single molecular unit, however DAT and other monoamine transporters may exist as oligomers. Early radiation inactivation studies using rat synapotosomes, which estimated the size of the transporter unit, found DAT size was larger than the estimated 70 kDa, suggesting DAT dimerization (Berger et al. 1994). Chemical crosslinking, fluorescence resonance energy transfer (FRET) microscopy, and co-immunoprecipitation studies confirmed DAT homo-oligmerization (Hastrup et al. 2001, Sorkina et al. 2003, Torres et al. 2003a), similar to SERT, GAT1, and NET (Kilic and Rudnick 2000, Schmid et al. 2001, Scholze et al. 2002). Cystine crosslinking studies determined DAT exists on the cell surface as a dimer of dimers, with the extracellular interfaces of TM6 and TM4 forming symmetrical interactions with other DAT units (Hastrup et al. 2003). Mutational analysis of the leucine zipper

region in TM2 suggested this region was also involved in a dimer interface (Torres et al. 2003a). However, recent crystal structure analysis found TM 2, 4, and 6 are part of the transporter core, and may not be readily accessible to extensive contacts with other transporters (Yamashita et al. 2005, Indarte et al. 2008). Furthermore, a recent study using substituted cystetine accessibility method (SCAM) found the leucine zipper in TM2 may not be involved in dimerization, but actually plays an indirect role in cocaine binding with its interaction with TM1 and TM6, areas involved in substrate binding (Sen et al. 2005). Yamashita et al. (2005) suggest the more peripheral TM11 and TM12 are likely candidates involved in oligomerization, similar to observations of SERT oligomers (Just et al. 2004).

While the role of oligomerization on transporter function is unclear, FRET studies of human DAT have shown that the transporter exists in oligomers in all cellular compartments occupied by DAT, and may be required for trafficking (Sorkina et al. 2003). Recent studies have also suggested substrates that act on the transporter, including dopamine and amphetamine, may modulate oligomerization after post-translational processing (Chen and Reith 2008). The researche's noted that the substrates dissociated dopamine transporter oligomers after endocytosis. Inhibition of endocytosis halted the dissociation of DAT complexes. These studies not only indicate the possible regulation of oligomerization, but also suggest oligomerization plays a role in endocytic trafficking and / or recycling of the transporter.

1.3 Effects of Drugs of Abuse on DAT

The reinforcement of drugs of abuse is partly mediated through their actions on the dopaminergic system. Several of these drugs are known to target the dopamine transporter, and interfere with DA uptake. This modulation of uptake alters synaptic DA levels in the brain, contributing to the psychoactive and addictive effects of the drugs. DAT is the primary site of action for psychostimulants, such as cocaine and amphetamine. However, the effect of alcohol on the transporter is under debate.

1.3.1 Psychostimulant action on DAT function

Pyschostimulants are known to mediate their effects by altering dopamine uptake. Cocaine, a potent competitive inhibitor of DAT, causes an accumulation of DA and overstimulation of DA receptors within the synapse (Nestler 2005). In the DA neuron-rich mesolimbic brain regions, the excess DA elicits states of euphoria and pleasure. Repeated use produces loss of control and compulsive response to drug related cues (Hyman et al. 2006). The importance of the transporter in the reinforcing properties of cocaine has been observed in animal models. DAT knockout mice, in a cocaine self-administration paradigm, have a significantly attenuated response and a decreased preference to cocaine (Giros et al. 1996). These findings were supported by evidence from transgenic mice that express a cocaine-insensitive DAT. The reinforcing effects of the drug were

completely abolished in these transgenic animals compared to mice expressing the wild-type form of the transporter (Chen et al. 2006).

The identification of DAT as the 'binding receptor' of cocaine was well established prior to our understanding of the transporter structure (Ritz et al. 1987). Site-directed mutagenesis identified residues in TM1 (D79 and W84) and TM6 (D313) as critical residues involved in the binding of cocaine (Chen et al. 2001, Ukairo et al. 2005). With the information gained from the $LeuT_{Aa}$ crystal structure, it was suggested that cocaine and DA binding sites overlap in the core of the transporter (Beuming et al. 2008). However, other studies indicate cocaine binding occurs on TM regions involved in DA binding, but in a more extracellular location, possibly blocking the DA translocation pathway (Vaughan et al. 2007). The recent computational model of DAT, suggesting a secondary docking site for transporter substrate and inhibitors, supports this idea (Indarte et al. 2008).

While cocaine exerts its effects on DAT by competitive inhibition, amphetamine increases synaptic DA concentrations by acting as a transporter substrate and inducing a reverse transport of the neurotransmitter (Fischer and Cho 1979, Sulzer et al. 1993, Sulzer et al. 1995, Sulzer et al. 2005). Amphetamines are widely abused; however, they have also long been used in the pharmacological management of several neurological disorders such as ADHD (Zaczek et al. 1991). Although the exact mechanism of transmitter efflux is unknown, however, evidence suggest amphetamine enters the cell as a DAT substrate, interacting with residues within the DA substrate-binding site (Beuming

et al. 2008). Upon cellular entry, the drug releases vesicular stores of DA, promoting reverse active transport via an inward-facing DAT (Zaczek et al. 1991, Fon et al. 1997, Erreger et al. 2008). Delineating the mechanism of action and binding of psychostimulants on the transporter aides researchers efforts in the development of pharmacological treatments for addiction, depression, and other neurological diseases.

1.3.2 Ethanol action on DAT

Alcohol (ethyl-alcohol; ethanol) is one of the most common drugs of abuse in society, and exerts its effects on several neurotransmitter systems, including dopamine. Like psychostimulants, ethanol induces an increase in synaptic DA, which is thought to mediate the reward and reinforcing effects of the drug (Gonzales et al. 2004). It has been established ethanol increases synaptic dopamine concentrations by inducing an increase of firing of DA neurons (Brodie et al. 1990, Weiss et al. 1993, Yim et al. 1997, Brodie and Appel 1998, Yim et al. 1998). As described above, several groups have also linked a polymorphism in the 3' UTR of the DAT1 gene to alcoholism and/or to the severity of the symptoms of alcohol withdrawal (Sander et al. 1997, Schmidt et al. 1998, Kohnke et al. 2005, Le Strat et al. 2008). However, the effects of ethanol on DAT function are controversial.

Dopamine release and synaptic concentration *in vivo* are commonly measured by electrochemical methods, including microdialysis, voltametry, and

chronoamperometry. Researchers have utilized these methods to also measure DA clearance in awake and anesthetized animals. The no-net-flux microdialysis, which has been reported to quantitatively measure transporter activity, have suggested the ethanol-induced increases in rat NAc is not due to an inhibition of DAT activity (Yim and Gonzales 2000). Further *in vivo* investigation of transporter activity by voltametry or chronoamperometry has suggested acute ethanol increases (Wang et al. 1997, Sabeti et al. 2003), decreases (Robinson et al. 2005), or has no effect (Budygin et al. 2001, Mathews et al. 2006) on DAT activity. Recently, chronic ethanol has been shown to increase DA uptake in rat NAc (Budygin et al. 2007).

The *in vitro* effects of ethanol on DA uptake suggest chronic and acute exposure potentiates DAT-mediated DA uptake. An increase in DA uptake and surface expression was reported in rats under chronic ethanol exposure (Rothblat et al. 2001, Carroll et al. 2006). When DAT is expressed in Xenopus oocytes, acute ethanol exposure increased DA uptake and transporter-associated currents in a time- and concentration-dependent manner (Mayfield et al. 2001, Maiya et al. 2002). Furthermore, surface binding with a radioligand cocaine derivative, suggested ethanol induced an increase in the number of transporters on the cell surface (Mayfield et al. 2001). Results from these studies suggest ethanol potentially alters DAT function by modulating transporter trafficking.

Acute ethanol exposure can also alter other neurotransmitter transporters. When expressed in HEK-293 cells, glycine transporters (GLYT1 and 2) are differentially affected by high concentrations of ethanol. Ethanol enhances GLYT1 function, while GLYT2-mediated uptake is inhibited (Nunez et al. 2000). Exposure to acute ethanol also enhances SERT uptake in synaptosomes prepared from rat cortical brain regions (Alexi and Azmitia 1991). The closest related transporter to DAT in structure and function, NET, has been shown in several studies *in vitro* to be inhibited by acute ethanol (Lin et al. 1993, Lin et al. 1997, Maiya et al. 2002). The opposing effects of acute ethanol on DAT and NET led researchers to identify ethanol-sensitive sites on DAT by generating chimeras between the two transporters (Maiya et al. 2002). These studies revealed IL1, which only differs in NET and DAT by four amino acids (DAT residues; F123, G130, I137, L138), was a critical area involved in the ethanol sensitivity of DAT (Figure 1.1.B). Of these residues, G130 and I137 appear to mediate the ethanol sensitivity of DAT. Mutation of either residue to the corresponding NET residue abolished DAT sensitivity to ethanol while maintaining the transporter's function when expressed in Xenopus oocytes. However, the exact mechanism of ethanol action on DAT is unclear.

1.4 DAT Functional Regulation

Redistributing the number of transporters expressed on the cell surface primarily regulates DAT function. This dynamic trafficking of DAT occurs in a

constitutive or regulated manner, and allows for rapid removal of transmitter. The transporters recycle between endosomal pools, which are localized near the plasma membrane, and the cell surface. Various steps in the DAT trafficking pathway can be regulated by second messenger systems, substrate, and pharmacological modulation.

1.4.1 Constitutive DAT trafficking

DAT proteins are synthesized and processed in the endoplasmic reticulum (ER) and Golgi complex. The C-, but not N-, terminus of the transporter is critical for export from the ER (Torres et al. 2001, Miranda et al. 2004). An interaction between the last three amino acids in the C-terminus of the transporter (LKV) and the PDZ binding domain of the PICK1 (protein that interacts with C kinase), an adaptor protein, has been shown to be important in ER-to-plasma membrane trafficking and subcellular localization of the transporter (Torres et al. 2001). However, others have found that while the C-terminal tail is crucial for subcellular targeting of the transporter, the PDZ domain of DAT and the PICK1 interaction is not required, nor was the interaction confirmed in a recent analysis of the DAT trafficking proteome (Bjerggaard et al. 2004, Maiya et al. 2006).

After delivery to the cell surface, DAT is internalized by a clathrin / dynamin-dependent mechanism into endosomal pools, involving the clathrin adaptor proteins epsin, Eps15 (epidermal growth factor pathway substrate clone 15) and Eps15R (Eps15-related protein) (Daniels and Amara 1999, Sorkina et al.

2005, Sorkina et al. 2006). DAT lacks classical intenalization signal motifs such as tyrosine- or dileucine-based sequences. However, a recent chimera study producing gain-of-function mutants between the N- and C- terminal tails of DAT and the transferrin receptor (TfR) or interleukin 2α receptor, respectively, suggested a constitutive endocytic signal in the C-terminus of the transporter (Holton et al. 2005). This nonclassical signal motif, which contains DAT residues 587-596, is conserved in other monoamine transporters (GAT1, SERT, and NET). The C-terminus also contains an overlapping protein kinase C (PKC) - sensitive endocytic signal (Holton et al. 2005, Boudanova et al. 2008b).

Studies of transporter internalization, induced and accelerated by the activation of PKC by phorbol 12-myristate 13-acetate (PMA), suggest DAT localized in endosomal pools can be targeted to degradation in lysosomes by ubiquitylation of lysine residues in the N-terminus (Miranda et al. 2005). Ubiquitylation is mediated by the E3 ubiquitin ligase, Nedd4-2 (neural precursor cell expressed, developmentally downregulated 4-2) (Sorkina et al. 2006). While it is believed DAT can be constitutively ubiquitinated and degraded in lysosomes, ubiquitin-mediated trafficking has only been examined in PMA-induced PKC activated cells (Sorkina et al. 2006). Early observations of DAT internalization in MDCK cells suggested internalized transporters are targeted to the lysosomal degradation pathway (Daniels and Amara 1999). However, several other studies have shown the majority of DAT actually remains in early endosomal pools, and

recycles back to the cell surface (Melikian and Buckley 1999, Loder and Melikian 2003).

Endosomal (or endocytic) recycling of the transporter allows for the rapid and efficient fine-tuning of dopaminergic signaling (Figure 1.2). Basal recycling rates are relatively modest, with a half-life ($t^{1/2}$) of expression on the cell surface of ~13 minutes, suggesting a rate of ~3-5% of DAT internalized per minute in heterologous cell expression lines (Loder and Melikian 2003, Li et al. 2004). These rates are comparable to constitutive recycling rates of GAT1 (Deken et al. 2000, Wang and Quick 2005). Analysis of endogenous cystine residues in the second extracellular loop suggest this area may play a key role in transporter expression on the cell surface (Wang et al. 1995). However, little is known of residues, protein-protein interactions, and/or cell signaling pathways involved in DAT insertion onto the membrane during constitutive recycling.

1.4.2 Regulated DAT trafficking

Regulation of dynamic trafficking of DAT plays a key role in facilitating the regulation of dopaminergic signaling (Mortensen and Amara 2003). Cell surface DAT levels can be acutely modulated by several second messenger pathways or pharmacologically by various drugs, including psychostimulants. These modulators alter specific steps or multiple steps of the endosomal recycling pathway (insertion and/or internalization) resulting in changes in transporter surface localization. Emerging evidence from these studies investigating intrinsic

or pharmacologically regulated transporter trafficking has also provided additional information on the cellular mechanisms involved in the DAT recycling pathway.

1.4.2.1 Regulation of DAT by kinase activity

The activation of several kinases, via second messenger systems, has been shown to alter DAT functional regulation. The regulation of DAT trafficking by phorbol ester-activated PKC has been well documented, and has been consistently shown to reduce the activity of the transporter in several transfected cell lines and synaptosomal preparations (Zhu et al. 1997, Daniels and Amara 1999, Melikian and Buckley 1999). PKC-mediated inhibition of DA uptake is a result of a downregulation of DAT surface expression, and not a change in transporter activity (Daniels and Amara 1999). The surface downregulation occurs by a PKC-induced increase in internalization by a clathrin/dynamin dependent mechanism and a parallel attenuation in transporter insertion into the membrane (Loder and Melikian 2003). Endocytosis of the transporter is accelerated by PKC activation, but is not required for constitutive internalization of DAT (Granas et al. 2003). Deletion of the N-terminus of the transporter abolishes PKC-induced regulation, but has no effect on DAT-mediated DA uptake.

The exact mechanism of PKC action on DAT is unclear. Evaluation of the DAT sequence suggests the presence of several consensus phosphorylation sites for PKC, PKA, and Ca^{2+} calmodulin kinase (Giros et al. 1991, Kilty et al.

1991, Shimada et al. 1991, Usdin et al. 1991). However, while PKC activation increases DAT phosphorylation (Vaughan et al. 1997), mutagenesis analysis showed the consensus sites were not essential in mediating PKC-induced regulation of DAT (Chang et al. 2001). These results suggest PKC-mediated functional regulation occurs in an indirect manner involving other accessory proteins or clathrin / dynamin activation pathways to induce internalization.

The PKC pathway has also been proposed to play a role in the molecular actions of amphetamine on DAT. Exposure to PKC inhibitors blocked amphetamine-induced efflux of DA in rat striatal slices in a DAT and Ca^{2+}-dependent manner (Kantor et al. 1999). When injected directly into rat NAc, PKC inhibitors also blocked amphetamine-mediated locomotor behavior (Browman et al. 1998). However, a recent study investigating amphetamine-induced trafficking of the transporter in stably expressing PC12 cells suggested the effects of the psychostimulant were independent of PKC activity (Boudanova et al. 2008a). Mutation of residues in DAT required for PKC-stimulated internalization had no effect on amphetamine-related alterations in transporter trafficking. These studies suggest amphetamine alters DAT trafficking in a direct manner, and will be discussed further in the next section.

In addition to regulation by PKC, other kinase signaling cascades have also been shown to regulate DAT-mediated DA uptake. Stimulation of the adenylyl cyclase-cyclic AMP (cAMP)-PKA pathway by forskolin or cAMP analogues potentiate DA uptake in rat striatal synaptosomes (Batchelor and

Schenk 1998, Page et al. 2000). Inhibition of phosphatidylinositol-3-kinase (PI 3-K) or mitogen-activated protein kinase (MAPK), which can be activated downstream of PI 3-K, decreased DA uptake and cell surface expression of DAT in rat striatal synaptosomes and transfected HEK-293 cells (Carvelli et al. 2002, Moron et al. 2003). Recent studies investigating the MAPK phosphatase (MKP3) action on the transporter activity suggested the phosphatase enhances DAT activity by possibly blocking dynamin-dependent internalization (Mortensen et al. 2008). Results discussed in this section suggest phosphorylation events may be important in regulation of the transporter, however the requirement of phosphorylation in all DAT regulation remains unclear.

1.4.2.2 Pharmacological and substrate-induced regulation of DAT

Therapeutic drugs and drugs of abuse have also been shown to modulate DAT function by altering trafficking of the transporters. The effects of several of these drugs, such as amphetamines, are mediated by acting as DAT substrate. Amphetamine is often prescribed for adult ADHD; however the psychostimulant is commonly abused, and can alter normal dopaminergic signaling by its actions on the transporter. DAT substrates, DA and amphetamines, have been shown to regulate transporter expression on the cell surface. Both substrates decrease DAT-mediated DA uptake by causing an increase in internalization of the transporter in cell expression systems and rat striatal synaptosomes (Saunders et al. 2000, Chi and Reith 2003). Results from *in vivo* voltammetry experiments

have also shown a decrease in transporter mediated DA clearance by DA and amphetamine (Gulley and Zahniser 2003).

Amphetamines act to block uptake of the endogenous substrate, and promote DA efflux, as described above (Zaczek et al. 1991, Fon et al. 1997, Beuming et al. 2008, Erreger et al. 2008). These actions are also mediated by modulating DAT endosomal trafficking. After an initial rapid delivery (< 3 minute) to the cell surface (Johnson et al. 2005, Furman et al. 2009), amphetamine has been shown by several studies, *in vivo* and *in vitro*, to cause DAT to sequestered in intracellular compartments (Sandoval et al. 2000, Sandoval et al. 2001, Kahlig et al. 2004, Kahlig et al. 2006). Amphetamine-regulated trafficking results in a suppression of transporter insertion and stimulation of endocytic rates, causing an overall decrease in surface levels of DAT (Boudanova et al. 2008a). The psychostimulant had no effect on global endosomal recycling or PKC-mediated regulation. The overall effects of amphetamine are elevated extracellular DA levels, which allows for continued receptor stimulation.

Other psychostimulants and therapeutic agents, including cocaine, bupropion (Wellbutrin), and methylphenidate (Ritalin) elevate synaptic DA levels by acting as an inhibitor of DAT (Dwoskin et al. 2006, Zahniser and Sorkin 2009). Cocaine and methylphenidate increase the number of DAT binding sites on the cell surface (Zahniser and Sorkin 2009). Cocaine has also been shown to elevate transporter levels on the cell surface directly in transfected cell lines using immunofluorescence and biotinylation (cell surface protein labeling) (Little et al.

2002). Consistent with the upregulation of surface localization, DA uptake kinetics suggested acute cocaine exposure increased uptake velocity (V_{max}) without altering DA binding affinity of the transporter (K_M) in rat NAc synaptosomes, and increased DA clearance in the striatum of anesthetized rats (Daws et al. 2002). These results suggest cocaine, and perhaps other DAT inhibitors, alter DAT trafficking, however it is yet to be determined if elevated surface levels of the transporters are due to an increase in insertion or a decrease in internalization.

Although a considerably large number of studies have investigated the actions of psychostimulants on DAT, less is known of the effects of some of the more commonly abused drugs such as nicotine and ethanol. Nicotine increases synaptic DA levels by activation of nicotinic acetylcholine receptors (nAChR). However, there is also evidence nicotine alters DAT function (Hart and Ksir 1996). Recent biotinylation and electrochemical studies investigating nicotine's mechanism of action on DAT regulation suggest nicotine increases DAT surface expression in the prefrontal cortex, but not striatum, via a nAChR-dependent mechanism (Zhu et al. 2009). The mechanism of nicotine-induced DAT surface localization has yet to be determined.

Ethanol is known to modulate the dopaminergic system, mediating the rewarding and reinforceing effects of ethanol. However, the action of ethanol on DAT is controversial, as described above. Chronic exposure to ethanol alters DAT binding density in human alcoholics experiencing relapse, ethanol preferring

monkeys, and rat striatal brain tissue (Tiihonen et al. 1995, Mash et al. 1996, Jiao et al. 2006). Less is known of the acute action of ethanol on DAT. Several in vitro studies in DAT expressing Xenopus oocytes suggest ethanol potentiates transporter function, and this increase in uptake is accompanied by an increase in DAT binding density (Mayfield et al. 2001, Maiya et al. 2002). However, others have found ethanol effects on the transporter are mediated through a neural-specific pathway (Ho and Segre 2001). Direct evidence of ethanol action on DAT surface expression and regulation in mammalian systems has yet to be determined. Determining the mechanism of action of ethanol on DAT function and regulation is essential in understanding ethanol-induced changes on synaptic dopaminergic transmission.

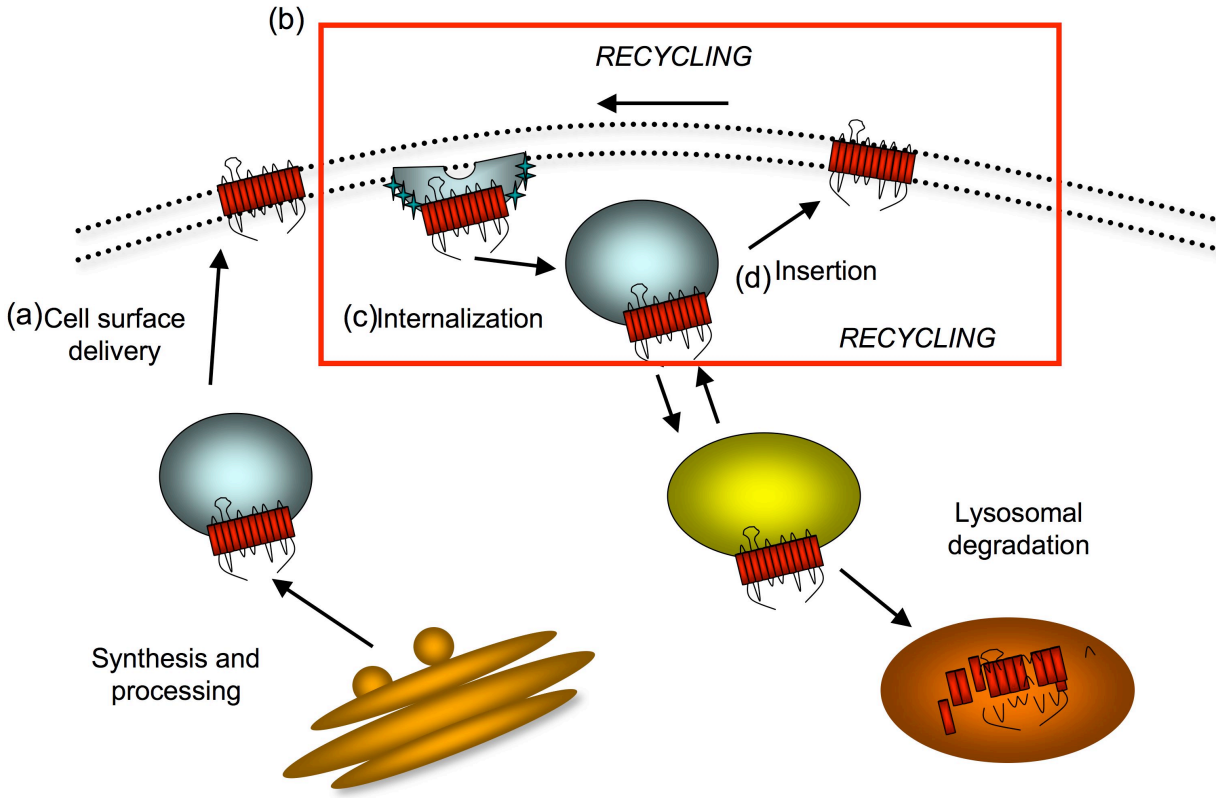

Figure 1.2: Intracellular DAT trafficking. After synthesis and post-translational processing, (a) transporters are delivered to the cell surface. DAT is internalized via dynamin- and clathrin-dependent mechanisms where they are predominantly localized to (b) endosomal recycling pools near the plasma membrane. Transporters are dynamically trafficked (c) to and (d) from the membrane to rapidly regulate extracellular dopamine level.

1.5 Research Aims

Electrophysiological and DA binding experiments in DAT expressing Xenopus oocytes suggest ethanol potentiates DAT-mediated DA uptake (Mayfield et al. 2001). The sensitivity of the transporter appears to be mediated by residues in the first intracellular loop of the transporter (Maiya et al. 2002). The absence of consensus sites for PKC or PKA phosphorylation in this loop

suggests ethanol may directly regulate transporter function through a traffcking-dependent mechanism. However, when expressed in neuronal or non-neuronal mammalian cell systems, others have suggested ethanol modulates DAT function though a neural-specific pathway (Ho and Segre 2001). We hypothesize ethanol potentiates DAT function in all mammalian cells by directly modulating transporter trafficking. Direct regulation of transporter function by ethanol may provide a rapid regulatory mechanism for ethanol-induced increases in synaptic DA concentrations. The objective of this study is to characterize ethanol effects on DAT function and regulation in mammalian cell systems and identify the mechanism of ethanol action on transporter regulation.

1.5.1 Aim 1: The effects of ethanol on DAT function in mammalian cell systems

Previous studies in DAT-expressing Xenopus oocytes suggest ethanol potentiates DAT function. The objective of this aim is to determine if similar changes are seen in neuronal and non-neuronal mammalian cell expression systems, and determine the specificity of ethanol action on the transporter. Mutant transporters (G130T), found to be insensitive to ethanol when expressed in Xenopus oocytes, will also be used as a tool to determine the specificity of ethanol action on the transporter. Dopamine upake and kinetics will be analyzed in the DAT-expressing cells to determine ethanol action on DAT function. Surface expression of DAT after ethanol exposure will be measured using cell surface

biotinylation assays. We hypothesize ethanol potentiates DAT function, and elevates DAT surface expression in a directly rather than through cell-specific pathways, in mammalian cell expression systems.

1.5.2 Aim 2: Ethanol action on DAT endosomal recycling

Preliminary experiments suggest ethanol enhances DAT cell surface expression. The objective of aim two is to determine the subcellular mechanism underlying ethanol-mediated regulation of DAT. DAT or G130T DAT (ethanol insensitive-mutant) will be stably expressed in HEK-293 cells, and surface expression will be compared to confirm ethanol-induced changes are trafficking mediated. The effects of ethanol on the DAT endosomal recycling pool size, and the rates of cell surface insertion and internalization will be measured using modified and reverse biotinylation assays. We hypothesize ethanol induces increases in DAT cell surface expression by altering the rates of endosomal recycling.

1.5.3 Aim 3: Characterization of the first intracellular loop of DAT in cell membrane trafficking

Chimera studies between DAT and NET in Xenopus oocytes suggest the first intracellular loop is critical in DAT trafficking and ethanol-mediated regulation of transporter trafficking. NET and DAT differ by four amino acids in the loop, DAT residues F123, G130, I137, and L138. Mutation of these residues to the

corresponding NET residues (IGLF DAT) abolished DA uptake and DA binding in Xenopus oocytes, suggesting the first intracellular loop is critical for DAT-specific trafficking. The objection of aim three is to characterize the function and cellular localization of the IGLF DAT in mammalian cells. Characterization of the IGLF mutant may be important in future studies identifying protein interactions critical in DAT trafficking. We hypothesize IGLF DAT uptake function will be abolished as a result of deficiencies in cell membrane trafficking.

Chapter Two: Materials and Methods

This chapter describes the materials and methods used to address specific aims one, two, and three presented in the following chapters.

2.1 Methods for Chapter Three (Specific Aim One)

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2.1.1 Site-directed mutagenesis

The ethanol insensitive mutant (G130T DAT) (Maiya et al. 2002) was constructed from wild-type human DAT cDNA subcloned in a pBK-CMV vector (Stratagene, La Jolla, CA) using the QuikChange site-directed mutagenesis kit (Stratagene) to replace the glycine at position 130 with a threonine. Both wild-type and G130T DAT cDNA constructs were subcloned into the mammalian expression vector pEGFP-C1, and transfected into HEK-293 or SK-N-SH mammalian cells. The constructs contained an enhanced green fluorescent protein (eGFP) marker to confirm DAT expression by visualization with fluorescent microscopy. Mutagenesis was verified by sequence analysis performed by the DNA Sequencing Core Facility at The University of Texas at Austin.

2.1.2 Cell culture and transient tranfections

HEK-293 and SK-N-SH cells were purchased from ATCC (Manassas, VA). Cells were maintained in a humidified atmosphere at 37°C and 5% CO_2 in HEK-293 growth media (Dulbecco's Modified Eagle Medium (DMEM) (Gibco, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) (Atlanta Biologicals, Lawrenceville, GA) and 0.1% Penicillin / Streptomycin (Sigma-Aldrich, St. Louis, MO)) or SK-N-SH growth media (Modified Eagle Medium (MEM) (Gibco, Carlsbad, CA) supplemented with 10% FBS and 0.1% Penicillin / Streptomycin). For uptake and kinetics assays, cells were split 24 hours prior to transfection into poly-D-lysine-coated 12-well plates (Becton-Dickinson, Bedford, MA) at a density of $0.5x10^5$ per well. Cells were transiently transfected with either eGFP wild-type human DAT or eGFP G130T DAT cDNA for 2 hours using Lipofectamine/PLUS reagents (Invitrogen, Carlsbad, CA). HEK-293 and SK-N-SH cells were transfected with 0.4 µg and 0.2 µg cDNA per well, respectively. Subsequent uptake or kinetics assays were performed 24 hours post-transfection. Unless noted otherwise, cells lines transiently expressing wild-type or mutated DAT cDNA were used for all experiments.

2.1.3 Stable Expression of Wild-type DAT

To generate monoclonal cell lines stably expressing wild-type DAT (DAT HEK cells), 70% confluent HEK-293 cells were transfected with 10 µg of eGFP wild-type human DAT cDNA for 2 hours using Lipofectamine/PLUS reagents

(Invitrogen). The transfected cells were split at 48 hours post-transfection into 100 mm cell culture dishes at a 1:10 dilution. G418 (1 mg/ml) (Sigma-Aldrich) antibiotic was added to the culture media 24 hours post-split for selection. After seven days of selection, G418-resistant colonies were plucked with cloning disks (Bel-Art, Pequannock, NJ), and the individual colonies were transferred into 24 well plates containing growth media. The colonies grew to 80% confluence, and those fluorescing eGFP were transferred to a larger culture flask for expansion. HEK-293 cells stably expressing eGFP wild-type DAT were maintained in growth media supplemented with 0.3 mg/ml G418 antibiotic.

2.1.4 [^{3}H] Dopamine uptake assays

HEK-293 or SK-N-SH cells were seeded in twelve well plates and transiently transfected with wild-type or G130T DAT as described above. Cells were then preincubated in serum-free media with or without ethanol (25, 50,100 mM) for 60 or 120min (3 wells per a condition). The ethanol pre-treatment media was quickly removed, and replaced with either the cell's respected growth media or KRH buffer (120 mM NaCl, 4.7 mM KCl, 2.2 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 10 mM HEPES, pH 7.4) containing 100 nM [^{3}H]DA and corresponding ethanol treatment (25, 50, 100 mM) for a 5 min incubation at 37°C to initiate [^{3}H]DA uptake. For the 5 min time point, cells were exposed to ethanol only during the uptake assay. Control cells, expressing wild-type or G130T DAT, were incubated in growth media containing 100 nM [^{3}H]DA without ethanol.

Uptake was terminated by quickly removing media, and washing the cells three times in 1 ml of ice-cold KRH buffer. Cells were then lysed with 500 µl of 1% sodium dodecyl sulfate (SDS) for 15 min at room temperature with gentle shaking. The whole-cell lysate was used to measure [^{3}H] DA uptake and protein concentration. Accumulation of [^{3}H]DA in the wild-type DAT or G130T DAT expressing cells was quantified in a liquid scintillation counter. Protein concentration (DC protein assay, Bio-Rad, Hercules, CA) was used to normalize the uptake assays, and nonspecific [^{3}H]DA uptake was determined by measuring uptake in the presence of the DAT inhibitor, cocaine (100 µM) (Sigma-Aldrich).

2.1.5 Kinetics assay

To determine the kinetics parameters (K_m and V_{max}) of DAT-mediated [^{3}H]DA uptake, HEK-293 cells were seeded in twelve-well plates (3 wells per condition), and transfected with wild-type DAT, as described above. Cells were treated with ethanol under maximal uptake conditions, determined by the dose and time dependent experiments (100 mM ethanol in serum-free media for 60 min). Kinetics of DAT-mediated dopamine uptake was evaluated by measuring uptake of [^{3}H]DA (100 nM) in the presence of increasing non-radioactive DA concentrations (0.025-1.5 µM) and in the presence or absence of ethanol (100 mM).

2.1.6 Cell surface biotinylation

Stably expressing DAT HEK cells were grown to 85-90% confluence in two T-150 culture flasks. The cells were washed twice with room temperature phosphate buffer saline (PBS), pH 7.4 (Gibco), and incubated at 37°C for 60 min with 10 ml of KRH buffer in the presence or absence of 100 mM ethanol. Cells were quickly washed twice with ice-cold PBS supplemented with 0.1 mM $CaCl_2$ and 1 mM $MgCl_2$ (PBS^{2+}), and incubated with 10 ml of 1.0 mg/ml sulfosuccinimidyl-2-(biotinamido)ethyl-1,3-dithiopropionate (EZ-link Sulfo-NHS-SS-Biotin) (Pierce, Rockford, IL) for 1h at 4°C. Excess biotin was quenched with two washes of ice-cold PBS^{2+} supplemented with 100 mM glycine, followed by two additional washes with ice-cold PBS^{2+}. Biotinylated cells were scraped into the final wash, collected by centrifugation at 1000xg for 5min, broken by ultrasonic sonication, and lysed in 0.5 ml of radioimmunoprecipitation assay buffer (150 mM NaCl, 10 mM Tris, pH 7.2, 0.1% SDS, 1.0% Triton X-100, 1% deoxycholate, 5 mM EDTA, and protease inhibitor cocktail) for 30 min on ice with intermittent agitation. The lysate was cleared of insoluble material by centrifugation at 10,000xg for 10min at 4°C, and 1:10 aliquot of the lysate was reserved to be analyzed later as total protein sample (total). The remainder of the cleared lysate was rocked overnight with 500 µl of UltraLink Immobilized NeutrAvidin Protein Beads (Pierce) at 4°C to separate biotinylated (cell surface) from non-biotinylated (intracellular) proteins via affinity chromatography. Unbound proteins were removed by 1000xg centrifugation at 4°C for 2 min. After

three washes with PBS, pH 7.4, the beads were incubated with 2x Laemmli buffer for 1 hr at room temperature while rocking to elute biotinylated (cell surface) proteins bound to NeutrAvidin beads. The protein concentration of the lysates was determined using DC Protein Assay kit (Bio-Rad, Hercules, CA), and an equal amount of protein was separated by SDS-PAGE in a 7.5% Tris-HCl Ready gel (Bio-Rad). After the proteins were transferred to polyvinylidene difluoride (PVDF) membrane, the blots were blocked overnight at 4°C in Tris-buffered saline (TBS-T) (10 mM Tris-Cl, pH 8; 150 mM NaCl; 0.01%Tween-20) containing 5% non-fat dried milk (Bio-Rad). DAT was detected with a rat monoclonal antibody specific for the N-terminus of DAT (MAB 369; Millipore-Chemicon, Billeria, MA) for 2 hr at room temperature while rocking in TBS-T containing 1% non-fat dried milk and 1% BSA. After three 10 min washes in TBS-T, blots were visualized using a horseradish peroxidase conjugated rat secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA) with enhanced chemiluminescence solution (Perkin Elmer, Boston, MA). Immunoreactivity was detected using Kodak Image Station 2000 mm, and band intensities were quantified with NIH Image J software. Blots were stripped and reprobed with antibodies against an intracellular marker, calnexin, an endoplasmic reticulum-resident protein. The absence of calnexin in the biotinylated protein sample indicates the integrity of the cell membrane was maintained during the biotinylation process, and only cell surface proteins were labeled.

2.1.7 Data Analysis

Specific uptake was calculated as the difference between total and non-specific uptake. Maximal ethanol potentiation was determined by pooling maximum [^{3}H]DA uptake data from each ethanol dose / time dependence experiments and calculating the mean for each cell type. Kinetic parameters (K_m and V_{max}) were determined by non-linear regression line analysis using Graphpad Prism, version 4.0 software. Ethanol-induced changes in DAT cell surface density was determined by quantifying the optical densities (OD) from the Western analysis of cell surface and total populations of the transporter. The relative amount of surface DAT was calculated by normalizing surface population OD to their respective total population OD of the transporter to obtain a ratio of surface expression. The ratio of surface expression in the ethanol treated sample was then normalized to the untreated ratio. The student's *T* test was used to compare ethanol treated samples to untreated controls, and one-way ANOVA with post-hoc analysis for dose and time comparisons.

2.2 Methods for Chapter Four (Specific Aim Two)

2.2.1 DNA constructs

cDNA encoding human DAT was obtained as previously described (Maiya et al. 2002). Mutant transporters were constructed from DAT cDNA subcloned into a pBK-CMV vector (Stratagene). Mutagenesis was performed using the

Quick Change site-directed mutagenesis kit (Stratagene) to replace glycine 130 with threonine (G130T). Both DAT and G130T DAT cDNA were subcloned into the mammalian expression vector pEGFP-C1 as described previously (Riherd et al. 2008).

2.2.2 Cell culture and stable transfections

Human embryonic kidney cells (HEK-293) (ATCC) were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum and 0.1% penicillin / streptomycin. HEK-293 cells were stably transfected with DAT or G130T DAT cDNA as previously described (Riherd et al. 2008). HEK-293 cells stably expressing DAT (DAT HEK cells) or G130T DAT (G130T DAT HEK cells) were cultured and maintained as previously described (Riherd et al. 2008). The constructs contained an enhanced green fluorescent protein (eGFP) tag to visually confirm transporter expression.

2.2.3 [^{3}H]DA Uptake Assays

Stably expressing DAT or G130T DAT HEK cells were seeded on poly-D-lysine coated 12-well plates at a density of 0.5 x 10^5 per well. Cells were incubated at 25°C in serum-free DMEM in the absence or presence of ethanol (10, 50, 100 mM) for 5, 30 or 60 min (three wells per condition). Uptake was initiated by adding 20 nM [^{3}H]DA during the final 3 min of the incubation time. Non-specific [^{3}H]DA uptake was determined using 100 μM PPP (S-(-)-3-(3-

hydroxyphenyl)N-propylpiperidinine hydrochloride; Sigma). Cells were washed three times in ice-cold Krebs-Ringer-HEPES buffer (120 mM NaCl, 4.7 mM KCL, 2.2 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 10 mM HEPES, pH 7.4) and lysed in 500 μl of 1% sodium dodecyl sulfate (SDS) for 15 min at room temperature with gentle shaking. Accumulation of [^{3}H]DA in the cells was quantified using liquid scintillation spectrometry (Beckman LS 6500; Beckman Coulter). Statistical significance was determined in Prism 4 by unpaired, two-tailed *t* test (basal uptake comparisons and individual time point comparisons to controls) and one-way ANOVA. Concentration- and time-dependent effects of ethanol on [^{3}H]DA uptake were calculated by post-hoc analysis for linear trend.

2.2.4 Biotinylation

To assess the effects of ethanol on DAT surface localization, DAT or G130T DAT HEK cells were seeded on tissue-culture treated 100 mm plates and grown to 80-90% confluence. Cells were incubated for 1 hr at 37°C in serum-free DMEM in the absence or presence of 100 mM ethanol. Surface proteins were biotinylated with 1 mg/ml biotin (sulfo-NHS-SS-biotin; Pierce) in PBS^{2+} for 30 min at 4°C as described above (Riherd et al. 2008). Biotinylated and non-biotinylated proteins were separated separated by incubation overnight at 4°C with pre-washed streptavidin M-280 Dynabeads (Invitrogen) on a rotating platform. Beads were washed three times with ice-cold PBS^{2+} containing protease inhibitor cocktail. Biotinylated proteins were eluted using 2x Laemmli buffer for 1 hr at

room temperature on a rotating platform. Total lysate and biotinylated proteins were analyzed with SDS-PAGE, immunoblotting and densiometry as previously described (Riherd et al. 2008). Blots were probed using a rat monoclonal antibody against the N-terminus of human DAT (MAB369, Chemicon) and a mouse monoclonal antibody against the endogenously recycled protein, human transferrin receptor (TfR) (13-6800, Zymed). To ensure the integrity of the plasma membrane was maintained during biotinylation, blots were also re-probed with a mouse monoclonal antibody against an intracellular marker, (CAL) calnexin (MAB3126; Chemicon), an endoplasmic reticulum protein.

2.2.5 Endosomal recycling pool measurment

To measure recycling pool size, cells were exposed to 1 mg/ml biotin in the absence or presence of 100 mM ethanol under trafficking permissive temperatures (37°C) for 30-90 min. Cells were solubilized in RIPA containing protease inhibitor cocktail. Biotinylated and non-biotinylated proteins were separated, and analyzed with immunoblotting as describe above.

2.2.6 Insertion Assays

Delivery of DAT to the plasma membrane was measured using a modified surface biotinylation assay at trafficking-permissive temperatures. First, surface proteins were biotinylated at 4°C (trafficking-restrictive) as described above. Excess biotin was removed, and cells were washed twice with ice-cold PBS^{2+}.

To initiate protein trafficking, cells were quickly washed with pre-warmed PBS^{2+} and incubated at 25°C in PBS^{2+} containing 1 mg/ml biotin ± ethanol (100 mM) for the desired time (*t*) (2, 5, 15 or 30 min). Additional biotinylated cells were kept in parallel at 4°C to measure basal DAT surface levels at *t*=0. The biotin reaction was quenched by two washes with ice-cold 100 mM glycine followed by two washes with ice-cold PBS^{2+}. Biotinylated and total lysate proteins were separated and analyzed by immunoblotting as described above. Insertion rate was calculated as described previously (Wang and Quick 2005). Briefly, biotinylated transporter was normalized to the respective total cellular DAT for each time point. Newly inserted transporters (T_t) were then measured as the increase in biotinylated DAT at each time point over basal DAT surface levels (T_0), and plotted over time. The rate was determined by fitting the time course of biotinylation to a single exponential equation to solve for τ: $T_t = T_0 + A[1\text{-}e^{(-t/\tau)}]$ where T_t = biotinylated DAT at time (*t*), T_0 =basal DAT surface levels, $A = T_t - T_0$, τ=time constant; Rate=$1/\tau$ (min^{-1}).

2.2.7 Internalization assays

Intracellular accumulation of the transporter and relative internalization rates were measured using a reversible biotinylation assay. DAT HEK cells were biotinylated at 4°C as described above, and the reaction was quenched with ice-cold 100 mM glycine. Cells were quickly washed with pre-warmed PBS^{2+} to initiate trafficking and incubated at 25°C in PBS^{2+} ± 100 mM ethanol for 2, 5, 15

or 30 min. Trafficking was rapidly halted by washing cells twice with ice-cold NT buffer (150 mM NaCl, 1 mM EDTA, 0.2% bovine serum albumin, 20 mM Tris, pH 8.6). Biotin remaining on the cell surface was cleaved using freshly prepared 50 mM TCEP (Tris(2-carboxyethyl)phophine hydrochloride; Pierce) in ice-cold NT buffer. TCEP was applied for 20 min at 4°C, removed and replaced with fresh TCEP for an additional 20 min incubation at 4°C. Biotinylated cells were kept in parallel at 4°C to measure total surface DAT (t=0). The stripping efficiency of the reducing reagent was determined by washing biotinylated cells with TCEP without allowing trafficking at 25°C. Cells were lysed, and biotinylated proteins were separated and collected using streptavidin Dynabeads as described above. The biotinylated fraction represented proteins internalized during trafficking. The total number of internalized transporter was calculated as the percent of biotinylated DAT (T_t) compared to total surface DAT (TS; T_0). The rate of internalization was determined using the same equation used to estimate insertion rate.

2.3 Methods for Chapter Five (Specific Aim Three)

2.3.1 Cell culture and stable transfections

Wild-type DAT and the IGLF mutant DAT (DAT residues replaced with cooresponding NET residues in the first intracellular loop, I137F, G130T, L138F, F123Y) (Maiya et al. 2002) were constructed from wild-type human DAT cDNA

subcloned in a pBK-CMV vector (Stratagene, La Jolla, CA) using the QuikChange site-directed mutagenesis kit (Stratagene), as described above. Mutagenesis was verified by sequence analysis performed by the DNA Sequencing Core Facility at The University of Texas at Austin.

Monoclonal cell lines stably expressing wild-type DAT or IGLF mutant DAT (DAT HEK and IGLF HEK cells, respectively) were generated, as described above. Briefly, 70% confluent HEK-293 cells were transfected with 10 μg of eGFP wild-type human DAT or eGFP IGLF DAT cDNA for 2 hours using Lipofectamine/PLUS reagents (Invitrogen). Stably expressing DAT or IGLF HEK cell colonies were selected with G418 antibiotic, plucked with cloning discs, and transferred to larger culture flasks for expansion. Stably expressing cell lines were maintained in growth media supplemented with 0.3 mg/ml G418.

2.3.2 [^{3}H] Dopamine uptake assays

Stably expressing DAT or IGLF DAT HEK cells were seeded on poly-D-lysine coated 12-well plates at a density of 0.5 x 10^5 per well. [^{3}H]DA uptake assays were prefomed as described above. Briefly, cells were incubated at 25°C in serum-free DMEM for 60min. Uptake was initiated by adding 20 nM [^{3}H]DA during the final 3 min of the incubation time. Non-specific [^{3}H]DA uptake was determined using 100 μM PPP. Cells were washed three times in ice-cold Krebs-Ringer-HEPES buffer, and lysed in 500 μl of 1% sodium dodecyl sulfate (SDS) for 15 min at room temperature with gentle shaking. Accumulation of

[^{3}H]DA in the cells was quantified using liquid scintillation spectrometry (Beckman LS 6500; Beckman Coulter). Statistical significance was determined in Prism 4 by unpaired, two-tailed *t* test.

2.3.3 Cell surface biotinylation

Cell surface levels of DAT or IGLF DAT was analyzed using the bioinylation method described above. Briefly, cells were seeded in T-150 flasks, and grown to 80-90% confluence. Cells were incubated for 1 hr at 37°C in serum-free DMEM. The cells were washed twice with ice-cold PBS^{2+}, and surface proteins were biotinylated with 1 mg/ml biotin in PBS^{2+} for 30 min at 4°C as described above (Riherd et al. 2008).

Chapter Three

The effects of ethanol on DAT function in mammalian cell systems

The following manuscript was published in *Alcohol*, "Ethanol potentiates dopamine uptake and increases cell surface distribution of dopamine transporter expressed in SK-N-SH and HEK-293 cells" *Alcohol*, (2008) volume 42, issue 6: 499-508. The manuscript is reprinted with permission from the publisher, Elsevier. David Galindo and Lucretia Krause performed assisted in uptake assays, and performed the kinetics assay. The remainder of the experiments were performed by D. Nicole Riherd Methner.

3.1 Introduction

Activation of the dopaminergic system in the mesolimbic areas of the brain controls a variety of physiological functions, including locomotion, emotion, and reward and reinforcement learning. The duration and strength of dopamine (DA) neurotransmission is dependent on the concentration of the neurotransmitter in the synaptic cleft. Re-uptake by the plasma membrane dopamine transporter

(DAT), located on the peri-synaptic terminal of dopaminergic neurons, predominately terminates dopaminergic neurotransmission and maintains homeostatic transmitter levels (Giros et al. 1991, Melikian and Buckley 1999, Gainetdinov and Caron 2003). DAT is a member of the Na^+/Cl^- family of neurotransmitter transporters that includes glycine, norepinephrine (NET), serotonin (SERT), and γ-aminobutyric acid (GAT1) transporters (Nelson 1998, Rudnick 1998). The transporters have similar topological arrangements and serve to terminate neurotransmission by removing transmitter from the extracellular space (Rudnick 1998).

Neurotransmitter transporters undergo rapid and dynamic trafficking to and from the cell surface in order to regulate neurotransmission. (Zhang et al. 1997, Melikian and Buckley 1999, Chi and Reith 2003, Li et al. 2004). The functional regulation of DAT, via alterations in the cell surface expression of the transporter, can occur through signaling cascades and exposure to DAT substrates. Phorbol ester activation of protein kinase C, tyrosine kinase inhibition, and N-glycosylation prevention decreases the transporter activity by down-regulating DAT surface expression (Vaughan et al. 1997, Zhang et al. 1997, Melikian and Buckley 1999, Li et al. 2004, Miranda et al. 2005, Hoover et al. 2007). When expressed in *Xenopus Laevis* oocytes, stimulation of the D_2 receptor induces a voltage-independent upregulation of DAT activity and [^{3}H]WIN 35428 surface binding, dependent on G(i/o) protein activation (Mayfield and Zahniser 2001). Similar studies in midbrain primary culture and human

embryonic kidney cells (HEK-293) reveal D_2 receptor stimulation increases DAT activity and surface expression via direct protein-protein interaction and ERK 1/2 pathways (Bolan et al. 2007, Lee et al. 2007). Stimulation of D_3 receptors expressed in HEK-293 cells also upregulates DAT activity and surface expression through MAPK and PI3K pathways (Zapata et al. 2007). Exposure to DAT substrates, inhibitors and other pharmacological reagents, such as psychostimulants can also alter DAT surface expression levels altering dopaminergic signaling in the brain.

Ethanol and psychostimulants such as cocaine and amphetamine induces cellular and molecular maladaptive changes in dopaminergic reward pathways, including alterations in DA release and/or DAT mediated DA uptake (Di Chiara and Imperato 1988, Koob and Weiss 1992, Zahniser and Doolen 2001, Zahniser and Sorkin 2004, Jayanthi and Ramamoorthy 2005, Hyman et al. 2006). Acute or chronic (or both) exposure to cocaine, d-amphetamine, and ethanol has been reported to alter DAT cell surface densities (Mayfield et al. 2001, Daws et al. 2002, Chi and Reith 2003, Gulley and Zahniser 2003, Zahniser and Sorkin 2004, Jayanthi and Ramamoorthy 2005, Johnson et al. 2005, Kahlig et al. 2006, Chen and Reith 2007). Cocaine rapidly increases DAT surface expression in mammalian cells and synaptosomes from rat striatum (Daws et al. 2002, Little et al. 2002, Chi and Reith 2003, Chen and Reith 2007), and competitively inhibits DAT-mediated DA uptake (Ritz et al. 1988). Other studies have shown chronic cocaine administration does not change the number of DAT binding sites, but

causes a dose-dependent reduction in DAT mRNA expression (Letchworth et al. 1997). Amphetamines also increase dopaminergic signal strength by reducing DAT-mediated DA uptake. D-amphetamine acts as a substrate for DAT and the vesicle transporter, VMAT2 causing competitive inhibition of DA binding, and reverse DA transport (Sulzer et al. 1993, Sulzer et al. 1995, Saunders et al. 2000, Johnson et al. 2005, Kahlig et al. 2006).

It is generally accepted that ethanol elevates synaptic DA concentrations by activating mesolimbic dopaminergic neurons (Imperato and Di Chiara 1986, Yoshimoto et al. 1992, Brodie and Appel 1998). However, ethanol's action on DAT is currently under debate. *In vitro* studies suggest ethanol has a potentiating effect on the transporter. After a 24 hour exposure, ethanol and cocaine increase DAT-mediated DA uptake both individually and combined in neuronal, but not non-neuronal, cell lines expressing the transporter (Ho and Segre 2001). These studies suggest ethanol and cocaine alters DAT function through a common neural pathway. However, electrophysiological and biochemical studies in transporter-expressing Xenopus *laevis* oocytes have also shown ethanol exposure potentiates DAT function and increases [^{3}H]WIN 35428 surface binding (Mayfield et al. 2001, Maiya et al. 2002). Two amino acids in the first intracellular loop of DAT, glycine 130 and isoleucine 137, mediate ethanol sensitivity of the transporter (Maiya et al. 2002).

In vivo studies examining the effects of chronic ethanol exposure on DAT suggest ethanol increases the number of DAT binding sites in alcohol-preferring

vervet monkeys (Mash et al. 1996), the striatum of violent alcoholics (Tiihonen et al. 1995), and in specific brain regions of Wistar-Kyoto rats. (Jiao et al. 2006). Chronic *in vivo* ethanol exposure has also been reported to potentiate DAT-mediated dopamine uptake (Carroll et al. 2006, Budygin et al. 2007). However, the acute action of ethanol *in vivo* is less clear. Electrochemical studies in mice and rats suggest ethanol has no effect on the transporter (Budygin et al. 2001, Budygin et al. 2005, Jones et al. 2006, Mathews et al. 2006). However, other electrochemical studies have shown acute ethanol either increases (Wang et al. 1997, Sabeti et al. 2003) or decreases dopamine uptake (Robinson et al. 2005).

The present studies aimed to investigate the hypothesis that short-term (≤ 2 hours) ethanol exposure alters DAT trafficking and increases cell surface distribution of DAT, potentiating transporter-mediated DA uptake in heterologous expression systems. We chose a neuronal mammalian cell line, catecholaminergic SK-N-SH neuroblastoma cells (Richards and Sadee 1986, Liu et al. 2001), and non-neuronal, HEK-293 cells, to express the transporter. Biochemical methods were used to characterize the effects of ethanol on transporter function and trafficking. We found ethanol potentiates DAT-mediated DA uptake in neuronal and non-neuronal cells expressing the transporter. This potentiation appears to be associated with an increase in expression of the transporter on the cell surface. Our results suggest ethanol has a direct mechanistic action in modulating DAT functional regulation in mammalian cell systems. These findings demonstrate ethanol's potentiating effect on the

transporter is consistent across multiple expression systems, and furthers our understanding of the molecular mechanisms of ethanol's action in mammalian systems.

3.2 Ethanol effects on dopamine uptake in transient DAT expressing SK-N-SH and HEK-293 cells

We examined ethanol actions on DAT function in SK-N-SH and HEK-293 cells expressing the transporter by [^{3}H]DA uptake assays. SK-N-SH and/or HEK-293 cells expressing wild-type or ethanol insensitive mutant (G130T) were pre-treated with ethanol (25, 50, 100 mM) for 0, 60 or 120 min, followed by an incubation in 100 nM [^{3}H]DA and corresponding ethanol treatment for 5 min at 37°C. Dopamine uptake was measured as the accumulation of [^{3}H]DA into DAT expressing cells. The 5 min time point includes cells exposed to ethanol only during the course of the uptake assay. Baseline dopamine uptake (untreated control cells) by SK-N-SH and HEK-293 cells expressing G130T DAT was not significantly different from the baseline uptake in cells expressing wild-type DAT (data not shown). Ethanol enhanced dopamine uptake in wild-type expressing SK-N-SH cells in a dose, but not time, dependent manner (Figure 3.1.A). The dose-response curve differed slightly from assay to assay, but a clear dose response pattern was observed for each individual assay (representative assay, 13, 27,and 30% potentiation for 25, 50, and 100 mM ethanol, respectively) (Figure 1A, *inset*). In SK-N-SH cells expressing the G130T DAT mutant, dose

response patterns also differed between assays (Figure 3.1.B); however, there was no dose or time response relationship observed in individual assays (representative assay, 27,14,and 18% potentiation for 25, 50, 100 mM ethanol, respectively) (Figure 3.1.B, *inset*).

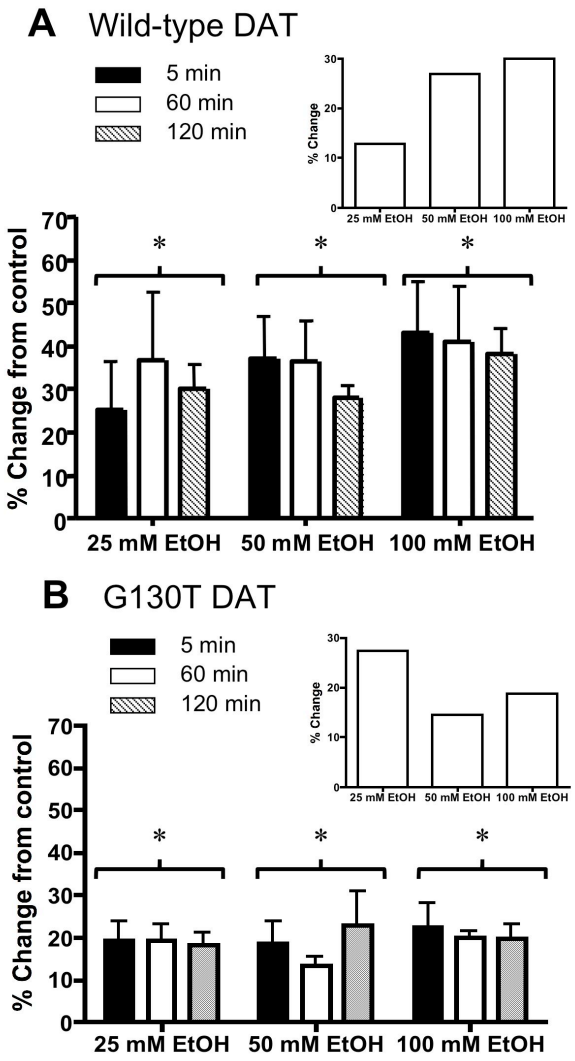

Figure 3.1: Dose and time dependent effects of ethanol pre-treatment on [^{3}H]DA (100 nM) uptake in DAT expressing SK-N-SH cells.. **A**. Wild-type DAT-mediated [3H]DA uptake. Ethanol significantly enhanced uptake compared to untreated controls for all pretreatment times, but not in a time dependent manner. (inset) Dose response relationship was variable between assays, but evident in individual assays. (representative 60 min ethanol pretreatment assay shown). **B**. G130T DAT-mediated [3H]DA uptake. Ethanol significantly enhanced uptake compared to untreated controls for all pre-treatment times, but not in a time dependent manner. (inset) Dose response relationship was variable between assays, and in individual assays (representative 60min ethanol pre-incubation assay shown). Data are expressed as means ± SEM of four experiments. *p < 0.01 compared with untreated control (one-way ANOVA for dose and time comparison tests).

Maximal ethanol effects (see methods) on transfected HEK-293 cells were similar to those seen in transfected SK-N-SH cells (Figure 3.2). For SK-N-SH

and HEK-293 cells expressing wild-type DAT, ethanol maximally potentiated dopamine uptake at 51% and 55%, respectively. In both cell lines expressing G130T DAT, ethanol significantly potentiated DAT-mediated [^{3}H]DA 29% in SK-N-S and 28% in HEK-293 (Figure 3.2). However, ethanol-induced potentiation of uptake was significantly reduced in G130T DAT expressing SK-N-SH and HEK-293 cells compared to cells expressing the wild-type transporter. As seen previously, the untransfected SK-N-SH cells exhibited a low-level of transporter activity that could be blocked by 100 μM cocaine (Liu et al. 2001), and these cells were potentiated 13% by ethanol compared to untreated, untransfected SK-N-SH cells, while untransfected HEK-293 cells exhibited no specific uptake (data not shown).

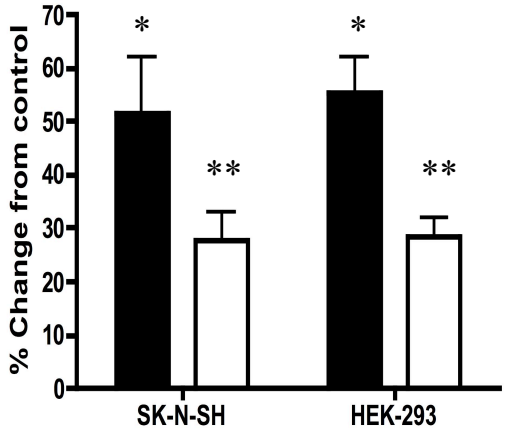

Figure 3.2: Maximum ethanol potentiation (see methods) from pooled [^{3}H]DA uptake data in SK-N-SH and HEK-293 cells expressing wild-type or G130T mutant DAT. Control cells, expressing wild-type or G130T DAT, were incubated in growth media with 100 nM [^{3}H]DA without ethanol. Ethanol significantly potentiated transporter-mediated [^{3}H]DA uptake compare to untreated controls in SK-N-SH cells expressing wild-type DAT or G130T DAT 51% AND 29%,respectively. Potentiation was significantly reduced in SK-N-SH cells expressing the G130Tmutant compared to wild-type DAT expressing cells. Ethanol potentiated transporter-mediated [^{3}H]DA uptake in HEK-293 cells expressing wild-type DAT or G130 DAT 55% and 28%,respectively. Potentiation was significantly reduced in HEK-293 cells expressing the G130Tmutant compared to wild-type DAT expressing cells. Data are expressed as mean ± SEM of maximal uptake values from three experiments. *$p < 0.05$ compared with untreated control.**$p<0.05$ compared with wild-type DAT (Student's *t* test).

3.3 Ethanol action on dopamine uptake kinetics

Kinetics parameters (V_{max} and K_m) of DAT-mediated [^{3}H]DA uptake were determined to further evaluate the mechanistic action of ethanol on the transporter. Cells were exposed to ethanol under maximal uptake conditions (100 mM ethanol for 60 min) determined by the dose / time dependent uptake assays described above. Ethanol enhanced maximal dopamine uptake velocity (V_{max}) 30-35% while the affinity for dopamine (K_M) remained unchanged (Figure 3.3).

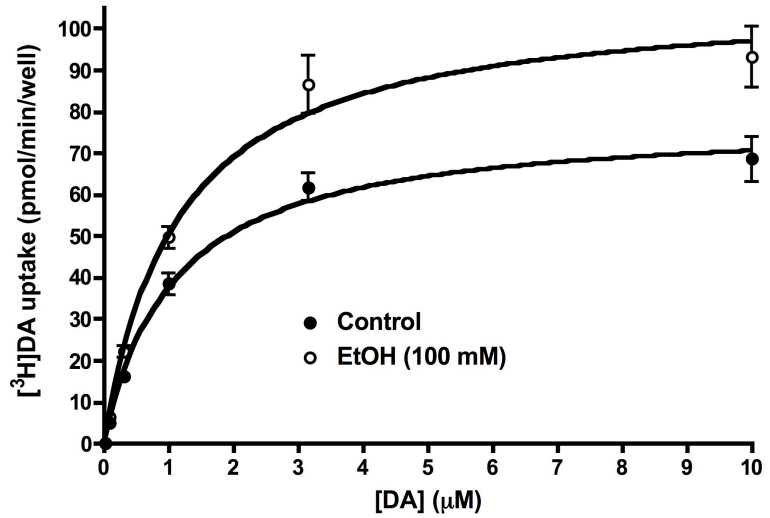

Figure 3.3: Ethanol pre-treatment (100 mM; 60 min) increased dopamine uptake velocity. Uptake of [3H]DA (10 nM-10 μM) was measured after a 60 min exposure to either KRH (control; fille circles) or ethanol (100 mM; open circles) in DAT expressing HEK-293 cells. Vmax (control 78.2 pmol/min/well; ethanol 107.9 pmol/min/well) and Km (control 1.06 μM; ethanol 1.12 μM) parameter estimates were obtained from the nonlinear regression curve fits. The graph is representative of four experiments. Each data point represents an average of three wells ± SEM.

3.4 Ethanol-induced changes of DAT cell surface localization

To investigate if the ethanol-induced increase in dopamine uptake (Figures 3.1 and 3.2) in DAT expressing mammalian cells was due to an increase in transporter expression on the cell surface, DAT surface density was examined by cell-surface biotinylation assays. Stably transfected HEK-293 cells from the same monoclonal populations were used in all biotinylation experiments to increase immunoblot signal intensity and reduce variance between experiments. HEK-293 cells adhere strongly to the surface of the flasks, reducing the loss of cells during the multiple washes of these biochemical experiments. Expression of total and cell surface population of wild-type DAT was determined in DAT HEK cells incubated in the presence or absence of ethanol (100 mM) for 1hr at 37°C (Figure 3.4.A). Total wild-type DAT was detected at ~55-125 kDa when assessed by anti-DAT (MAB 369; Chemicon). The presence of the eGFP tag on the N-terminal tail of the transporter increased the size ~25 kDa from the expected size of DAT (~55-90 kDa). The lower bands (~55-90 kDa) reflect lysosomal-degraded and unglycosylated DAT. The upper bands (~90-125 kDa) are glycosylated forms of the transporter, expected at the cell surface or in recycling pools of DAT. Ethanol (100 mM) had no effect on the total population of the transporter in cells; however, ethanol treatment significantly increased the surface density of DAT (Figure 3.4.B) by 40 - 50% (Figure 3.4B, *inset*). Ethanol had no effect on the expression of the intracellular endogenous control protein, calnexin (Figure 3.4.A). Less than 2% of calnexin was biotinylated (data not shown), indicating

the integrity of the cell membrane was maintained throughout the surface labeling process.

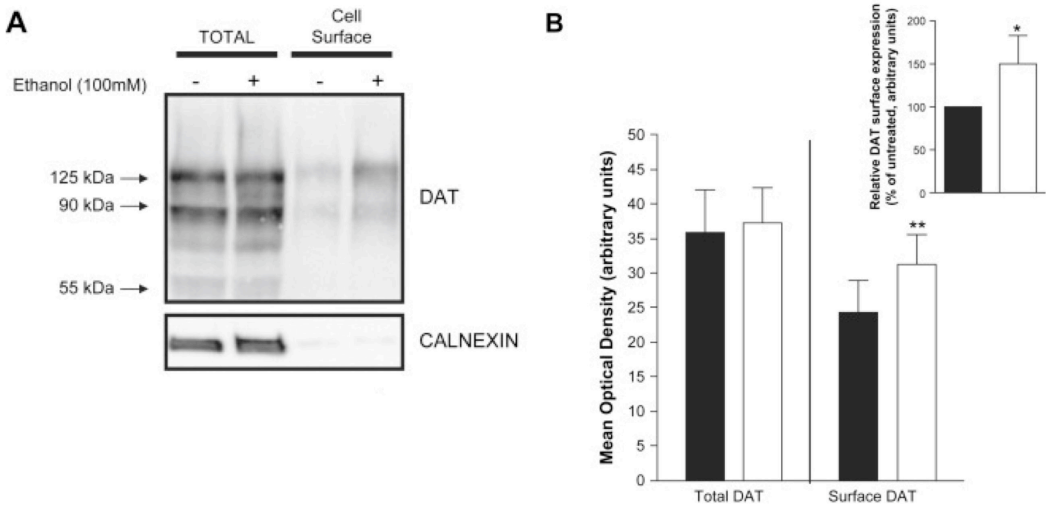

Figure 3.4: Ethanol increases surface density of DAT. Cells were incubated in KRH buffer in the absence or presence of 100 mM ethanol for 1h at 37°C. **A**. Representative immunoblots of DAT and calnexin in biotinylated DAT HEK cell lysate from four experiments. Shown are the total extracts (total DAT 55-125 kDa) and biotinylated extracts (cell surface DAT 90-125 kDa) from DAT HEK cells with indicated treatments. Blots were stripped and reprobed with the antibody for calnexin. **B.** Mean optical density (O.D.) of total DAT in ethanol-treated (empty bars) DAT HEK cells did not significantly change compared to total DAT in untreated cells (filled bars), while the cell surface DAT O.D. was significantly increased in ethanol treated cells. **p <0.01 compared to untreated surface DAT O.D. (Student's t test). (inset) Quantification of the percent change in cell surface DAT populations between ethanol treated and untreated cells. Ethanol significantly increases surface DAT populations 40-50% (expressed as a percentage relative to the total amount of DAT in the respective lysate (untreated or ethanol-treated) and normalized to untreated surface DAT). Data are expressed as normalized values ± SEM of four experiments. *p < 0.05 compared with untreated surface DAT population (Student's t test).

3.5 Discussion

The goal of this study was to determine the direct action of ethanol on DAT functional regulation in mammalian cell expression systems. We found a short-term ethanol exposure potentiates dopamine uptake in both mammalian neuronal (SK-N-SH) and non-neuronal (HEK-293) wild-type DAT expressing cells. This potentiation was significantly reduced in cells expressing the ethanol insensitive mutant, G130T DAT. Ethanol-induced enhancement of DAT activity was associated with an increase in transporter expression on the cell surface. Our results in mammalian systems are in agreement with similar biochemical studies examining the effects of ethanol on DAT functional regulation in other *in vitro* systems (Mayfield et al. 2001, Maiya et al. 2002). Ethanol alters the functional regulation of DAT across multiple expression systems, suggesting a direct mechanistic action of ethanol on the transporter trafficking system.

The reward and reinforcing effects of ethanol are predominately mediated through the mesolimbic dopaminergic system (Di Chiara and Imperato 1988, Carboni et al. 2000, Engleman et al. 2000, Katner and Weiss 2001, Tang et al. 2003b, Gonzales et al. 2004). Maladaptive changes in various properties of the dopaminergic system, including regulation of neurotransmission, contribute to ethanol addiction (Gonzales et al. 2004). Mammalian cell lines serve as important model systems to study the molecular mechanisms of drug and alcohol action. In this study, catecholaminergic SK-N-SH neuroblastoma and non-neuronal HEK-293 cells served as our mammalian cell model systems. Both cell

lines were transfected with human wild-type or ethanol-insensitive G130T DAT to detect the effects of short-term ethanol exposure on DAT function and cell surface expression. These studies allowed us to identify potential molecular mechanisms of ethanol action on the transporter.

We found ethanol exposure potentiates dopamine uptake in neuroblastoma SK-N-SH and non-neuronal HEK-293 cells expressing wild-type DAT. Ethanol increased DAT activity in a clear dose-dependent manner within each individual assay. The dose response relationship was more variable across assays. This variation was likely due to differences in transfection efficiencies, growth rates, or passage number. Previous studies examining the effects of a 24 hour exposure to ethanol and cocaine (individually or combined) in neuronal and non-neuronal cell lines resulted in an increase in DAT-mediated DA uptake only in neuronal cell lines (Ho and Segre 2001). Their results suggest ethanol and cocaine act on DAT through common neural-specific pathways to alter transporter function. We found ethanol alters DAT function in both non-neuronal and neuronal cell lines. This contradiction could be due the differences in ethanol exposure time and/or cell type. Our results suggest ethanol may have a direct mechanistic action on the transporter trafficking system. The SK-N-SH cell line used in these studies endogenously express NET and DAT. These transporters are responsible for a low level of uptake, which was not blocked during these assays. However, this endogenous uptake is negligible compared to the uptake by the wild-type or G130T DAT overexpressed in these cells, thus

any contribution of endogenous transporters to our reported effects is minimal. Our results demonstrating the enhancement of DAT activity by ethanol are consistent with previous electrochemical, chronoamperometry, and biochemical experiments in rats (Wang et al. 1997, Sabeti et al. 2003), and Xenopus oocytes (Mayfield et al. 2001, Maiya et al. 2002).

Chimera and site-directed mutagenesis studies in the Xenopus oocyte expression system identified the site of ethanol action at the first intracellular loop between transmembrane domains two and three of DAT. Specifically, a mutation of glycine 130 for threonine, a non-conservative amino acid change, eliminated the potentiating effects of ethanol on transporter action and cell surface binding in a Xenopus oocyte expression system (Maiya et al. 2002). As seen in oocytes, basal [^{3}H]DA uptake (0 mM ethanol) in SK-N-SH and HEK-293 cells expressing G130T DAT is similar to wild-type DAT. However, in the mammalian cell models the mutation reduced, but did not eliminate ethanol sensitivity. While the G130T mutant demonstrated a significant potentiation of function after ethanol exposure, potentiation was also significantly reduced by half compared to wild-type DAT expressing cells. This ethanol-induced increase in G130T DAT activity was not dose-dependent. The lack of a dose-response effect is not due to variance in transfection efficiency, growth rates, or passage number, because none of the individual assays demonstrated a dose-response pattern. It is more likely due to the reduced sensitivity of the DAT mutant to ethanol. Alternatively, ethanol could be acting as an allosteric modulator of DAT, as seen with the GABA(A) receptor .

This would suggest the G130T mutation disrupts a critical allosteric modulation site for ethanol. However, the potentiation of DAT function is associated with an ethanol-induced increase in surface expression of the transporter, as seen in the biochemical studies described above (Figure 3.4) and in oocytes (Mayfield et al. 2001, Maiya et al. 2002). We hypothesize the G130T mutation is disrupting a protein-protein interaction site critical for ethanol-regulated trafficking of DAT to the cell surface. The elimination of ethanol sensitivity in G130T DAT expressing oocytes was attributed to a lack of change in cell surface distribution of the transporters (Maiya et al. 2002). The differences in ethanol potentiation between oocytes and mammalian cells are is most likely due to differences in trafficking mechanisms and/or cellular machinery in the model systems. Studies are in progress to examine the differences in ethanol-induced trafficking of G130T versus wild-type DAT in mammalian cell expression systems.

Trafficking and alterations in surface distribution of DAT is the predominant mechanism to rapidly regulate transporter function (Jayanthi and Ramamoorthy 2005, Maiya et al. , Torres 2006). Psychostimulants such as cocaine and amphetamine have been shown to modulate DAT activity by redistributing the transporter to the cell surface. Our kinetics data demonstrated DAT activity was potentiated by ethanol (increased V_{max}) with no change in transporter binding affinity (K_m). The potentiation of uptake velocity parallels an increase number of transporters on the cell surface in DAT HEK cells. While stably expressing DAT HEK-293 cells have been reported to have higher levels

of [^{3}H]DA uptake compared to transiently expressing cells (Chen and Reith 2007), our results are in agreement with other *in vitro* studies using DAT expressing Xenopus oocytes where potentiation of DAT activity by ethanol also correlated with an increase in [^{3}H]WIN 35428 surface binding (Mayfield et al. 2001). *In vivo*, ethanol has been reported to differentially modulate the expression of a variety of proteins in mesolimbic areas of the brain (Bell et al. 2006), including DAT (Tiihonen et al. 1995, Mash et al. 1996, Jiao et al. 2006). Abstinent alcohol-preferring vervet monkeys have an increase number of cell surface DAT, and chronic alcohol consumption down-regulates surface DAT expression (Mash et al. 1996). However, chronic ethanol has also recently been shown to increase DAT binding in specific brain regions of Wistar-Kyoto rats, including areas of the mesolimbic system involved in addiction (Jiao et al. 2006), while differential expression of surface DAT has been seen in violent versus non-violent alcoholics (Tiihonen et al. 1995). These changes could reflect ethanol-induced neuroadaptations in the brain, but further research is needed to compare expression differences between various brain regions, drinking models, and model systems.

Ethanol-mediated increases in DAT activity and cell surface expression after a short-term exposure, could represent a mechanism that underlies an acute functional tolerance to ethanol (Mayfield et al. 2001). Another amine transporter of the Na^+/Cl^- family, GAT1, has been shown to have an acute tolerance to ethanol (Byas-Smith et al. 2004). Although GAT1 does not directly

interact with ethanol, transporter activity increased in response to acute ethanol exposure. Interestingly, the DAT inhibitor, GBR 12909 and ethanol cross-sensitize in DBA/2J mice (Broadbent et al. 2005). These experiments suggest DAT inhibition unmasks increased basal levels of dopamine due to ethanol sensitization, which is normally compensated for by DAT uptake activity.

It is generally accepted ethanol exposure increases dopamine release in the mesolimbic areas of the brain (Yim and Gonzales 2000, Gonzales et al. 2004). However, ethanol action on DAT regulation is currently under debate. *In vivo*, chronic ethanol exposure has been shown to increase DAT-mediated uptake (Carroll et al. 2006, Budygin et al. 2007), while acute ethanol has been reported to increase (Wang et al. 1997, Sabeti et al. 2003), decrease (Robinson et al. 2005) or have no effect on DAT activity (Budygin et al. 2001, Budygin et al. 2005, Jones et al. 2006, Mathews et al. 2006). Electrochemical studies using the 'no net flux' microdialysis method suggested acute ethanol-induced increases in dopamine levels were not due to an inhibition in transporter function, but an increase in release (Yim and Gonzales 2000). *In vitro* electrophysiological and biochemical experiments in a Xenopus oocyte model system, which measured DAT activity in a more direct manner, suggested ethanol exposure increases DAT-mediated uptake through a redistribution of transporter expressed on the cell surface (Mayfield et al. 2001, Maiya et al. 2002). Many of the electrochemical studies described above indirectly examine transporter activity and regulation as a function of alterations in extracellular dopamine levels. The

contradiction between findings could be contributed to inherent differences between model systems and/or techniques.

In conclusion, we determined ethanol induced a potentiation of DAT activity in both DAT-expressing neuroblastoma SK-N-SH and non-neuronal HEK-293 mammalian cells. This potentiation appears to be associated with an increase in the number of transporters expressed on the cell surface. These findings are in agreement with similar biochemical experiments in other *in vitro* systems, suggesting ethanol may have a direct action on the transporter's trafficking system thus modulating DAT functional regulation. In this study, we established and characterized working mammalian cell lines for future mechanistic studies involving the transporter, and compared ethanol's effect on DAT function and regulation. These data further our understanding of the molecular mechanism of intracellular ethanol action, and provides insight to potential sites of maladaptive changes involved in alcoholism.

Chapter Four

Ethanol Action on Endosomal Recycling of Human Dopamine Transporters

The following chapter has been submitted for publication, and is currently under review as: Riherd Methner, D.Nicole and R. Dayne Mayfield “Ethanol alters endosomal recycling of human dopamine transporters.” 2009.

4.1 Introduction

Dopamine (DA), a major central nervous system neurotransmitter, is involved in reward and reinforcing behaviors. DA signaling relies on a critical balance between release and removal of the neurotransmitter within synaptic clefts. Drugs of abuse, including psychostimulants and ethanol, cause maladaptive changes in DA signaling in mesolimbic areas of the brain, leading to addictive behaviors. Localized on pre-synaptic dopaminergic terminals, the dopamine transporter (DAT) is responsible for terminating DA signaling by rapidly removing the transmitter from the synaptic cleft region (Torres et al. 2003b). DAT and other monoamine transporters, including norepinephrine, γ-amino butyric acid, and serotonin (NET, GAT1, and SERT, respectively), clear extracellular transmitter via a reuptake mechanism (Nelson 1998).

Regulation of DAT function is mediated by recycling of the transporters between intracellular compartments and the plasma membrane (Melikian and

Buckley 1999). This dynamic trafficking occurs in both a constitutive and regulated manner to increase or decrease the number of transporters on the cell surface that are available for transmitter reuptake. Trafficking modulators, such as activated protein kinase C (PKC), have been shown to alter basal transporter trafficking rates. PKC-mediated regulation causes an intracellular accumulation of DAT by increasing internalization and decreasing insertion of the transporter on the cell surface (Loder and Melikian 2003). In addition to intrinsic transporter modulators, various drugs of abuse are known to target monoamine transporters. DAT is the main site of action of several psychostimulants such as cocaine and amphetamines (Ritz et al. 1988, Sulzer et al. 1993, Jayanthi and Ramamoorthy 2005). Cocaine inhibited DAT and increased the number of transporters on the cell surface by altering DAT trafficking in rat striatum and heterologously expressing cells (Daws et al. 2002, Little et al. 2002, Chi and Reith 2003, Chen and Reith 2007). Amphetamine produced an initial increase in cell surface populations of the transporter in rat striatal synaptosomes (Johnson et al. 2005), followed by internalization of cell surface transporters and DAT-mediated DA efflux (Saunders et al. 2000, Kahlig et al. 2006, Binda et al. 2008, Boudanova et al. 2008a). Psychostimulant-induced modulation of transporter trafficking plays a critical role in mediating the mechanism of action of the drug.

Ethanol is also known to activate DA pathways in the reward and reinforcing areas of the brain. Many groups have shown that ethanol alters DA signaling by increasing release of the transmitter (Imperato and Di Chiara 1986,

Yoshimoto et al. 1992, Brodie and Appel 1998); however, recent *in vitro* studies from our lab suggest ethanol modulates DAT function as well. Experiments in DAT-expresssing Xenopus *laevis* oocytes have shown ethanol potentiated [^{3}H]DA uptake in a time- and concentration-dependent manner and enhanced transporter-mediated currents. The ethanol-induced increase in DA uptake in the oocytes was accompanied by an increase in radioligand [^{3}H]WIN 35,428 ([^{3}H]2-β-carbomethoxy-3β-(4-fluorophenyl)tropane) binding to DAT (Mayfield et al. 2001). Similar effects of ethanol on DAT function and surface localization were found when the transporter was expressed in neuroblastoma (SK-N-SH) or human embryonic kidney-293 cells (HEK-293) (Riherd et al. 2008). Ethanol potentiation, however, is not observed among other monoamine transporters. For example, ethanol inhibited uptake of the closely related NET (Lin et al. 1993, Maiya et al. 2002). The opposing effect of ethanol on DAT and NET led to identification of ethanol-sensitive sites in the first intracellular loop of DAT using chimeras between DAT and NET (Maiya et al. 2002). Specifically, when glycine 130 and/or isoleucine 137 were replaced with the corresponding NET residues, threonine and phenylalanine, respectively, the ethanol sensitivity of the transporter was abolished in oocytes (Maiya et al. 2002) and significantly reduced in SK-N-SH and HEK-293 cells (Riherd et al. 2008). The loss of ethanol sensitivity by these point mutations suggest ethanol alters DAT function or trafficking in a direct manner.

The ethanol-induced potentiation of DA uptake and increase in DAT cell surface expression suggest ethanol modulates transporter function by redistributing DAT to the cell surface. However, it is not known how or if ethanol directly regulates DAT trafficking. In this study, we used [^{3}H]DA uptake and biochemical methods to assess the pharmacological action of ethanol on DAT function and endosomal recycling. Basal and ethanol-regulated rates of insertion and internalization of the transporter were measured in HEK-293 cells using modified biotinylation assays. We found ethanol modulates DAT function by increasing the rate of insertion while having no effect on DAT endocytosis, resulting in an accumulation of the transporter on the plasma membrane. These results suggest an additional mechanism by which ethanol can potentially affect synaptic dopamine dynamics in mammalian systems.

4.2 Characterization of Ethanol Action on DAT and G130T DAT HEK Cells

We first characterized ethanol action on DAT function in HEK-293 cells stably expressing DAT. We also tested function of the ethanol-insensitive G130T DAT mutant. DAT or G130T DAT was transfected into HEK-293 cells and selected for stable expression. Stably expressing cells were incubated with 100 nM [^{3}H]DA for 3 min, and dopamine uptake was measured as the accumulation of [^{3}H]DA into the cells. The G130T DAT mutation had no effect on basal [^{3}H]DA uptake (Figure 4.1.A). Cells were pretreated with ethanol (10, 50, 100 mM) for 5, 30 or 60 min. Ethanol potentiated [^{3}H]DA uptake in DAT HEK cells in a

concentration-, but not time-dependent manner (Figure 4.1.B, upper panel). Compared to untreated DAT HEK cells, 100 mM ethanol produced a 66-77% increase in uptake at all time points. Ethanol had no significant effect on G130T DAT HEK mediated [^{3}H]DA uptake compared to untreated G130T DAT HEK cells (Figure 4.1.B, lower panel).

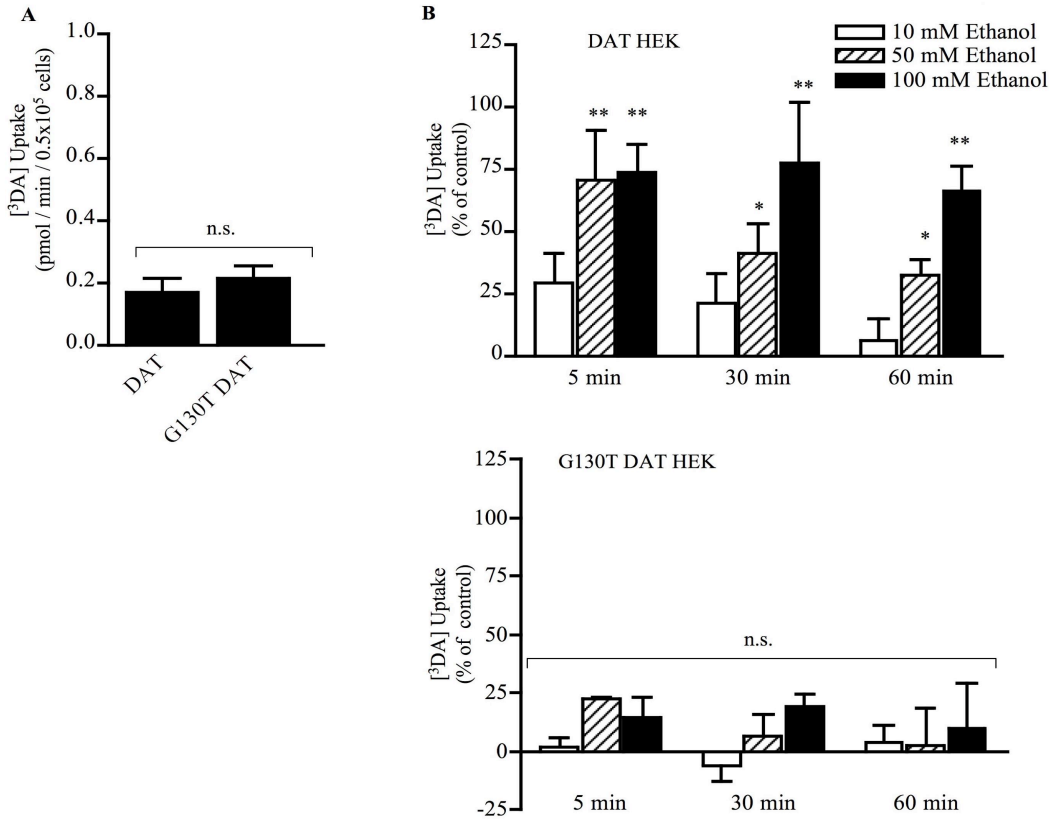

Figure 4.1: **Functional characterization of ethanol action on DAT and G130T DAT HEK cells.** Uptake of [^{3}H]DA was measured in stably expressing DAT or G130T DAT HEK cell cultures. **A.** Basal [^{3}H]DA uptake was unchanged (n.s.: not significant) by the G130T mutation. (unpaired *t* test; *n*=7 experiments with triplicate samples) **B.** Stably expressing DAT or G130T DAT HEK cells were exposed to ethanol (10, 50 or 100 mM) for 0, 5, 30 and 60 min at 25°C. *Upper panel*, Ethanol potentiated DAT-mediated [^{3}H]DA uptake in a concentration-dependent manner compared to controls (*p < 0.05 or **p < 0.01, one-way ANOVA with post-hoc analysis for linear trend; *n*=5 experiments per time point with triplicate samples). *Bottom panel*, The G130T DAT mutation abolished ethanol sensitivity of the transporter. (unpaired *t* test for individual ethanol concentration comparisons to controls; *n*=5 experiments per time point with triplicate samples. Values represent mean ± S.E.M.

4.2.1 Comparison of DAT and G130T DAT HEK surface localization after ethanol exposure

We have recently demonstrated that ethanol increases cell surface populations of DAT in HEK-293 cells (Riherd et al. 2008). However, the mechanisms underlying these changes were unknown. We first verified ethanol-induced changes on DAT surface populations by comparing its effect on DAT and G130T DAT surface localization. We used biotinylation assays to assess cell surface transporter levels in DAT HEK cells after 1 hr incubation with 100 mM ethanol at 37°C (see representative immunoblot, Figure 4.2.B). eGFP conjugated DAT or G130T DAT is ~70-120 kDa, depending on glycosylation state. Under control conditions, 30.4% ± 3.6 of the total DAT population was expressed on the cell surface, while ethanol-exposed cells were found to have 50.6% ± 4.4 of the total DAT population on the surface (Figure 4.2.B). In agreement with our previous study (Riherd et al. 2008), ethanol significantly increased DAT surface expression ~40-45% while having no effect on the total cellular population of the transporter (data not shown). To determine if ethanol has a global, non-specific effect on cellular trafficking, blots were re-probed for TfR, an endogenous protein that also undergoes endosomal recycling (Figure 4.2.A). Basal cell surface levels of TfR in DAT HEK cells were not significantly changed by ethanol (11.4% ± 1.5 compared to 11.6% ± 1.7 of total TfR for control and 100 mM ethanol-exposed cells, respectively) (Figure 4.2.B). We repeated the cell surface biotinylation experiments with G130T DAT HEK cells,

which were functionally insensitive to ethanol (see representative immunoblot, Figure 4.2.C). Ethanol had no significant effect on the number of mutant transporters on the cell surface (33.7% ± 1.9 compared to 29.5% ± 0.7 of total G130T DAT for control and 100 mM ethanol-exposed cells, respectively) or the number of TfR (13.3% ± 1.3 compared to 12.5% ± 2.3 of total TfR for control and 100 mM ethanol exposed cells, respectively) (Figure 4.2.D). DAT and G130T DAT immunoblots were re-probed with an antibody against the intracellular marker, CAL (Figure 4.2.A and C). The integrity of the plasma membrane was maintained throughout the course of the biotinylation assay (<2% of total calnexin was biotinylated; data not shown).

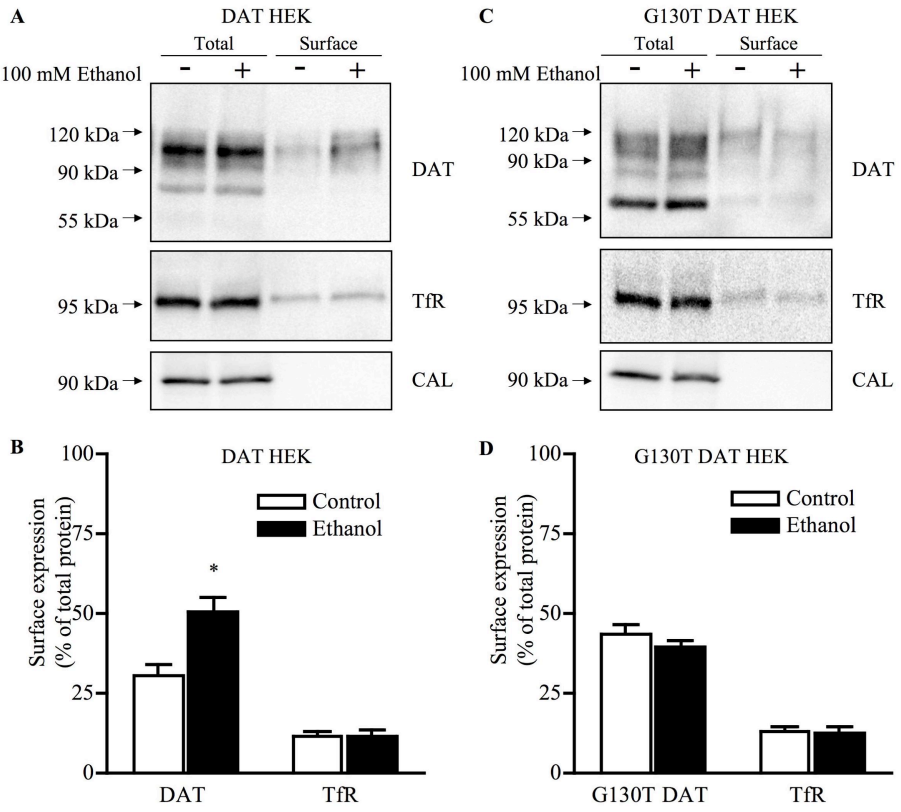

Figure 4.2: Ethanol promotes DAT surface localization. DAT and G130T DAT HEK cells were incubated for 60 min at 37°C ± 100 mM ethanol. Cell surface levels of the transporter were determined using surface biotinylation assays. (**A** and **C**) Representative immunoblots of total (Total) and biotinylated (Surface) populations of transporter, TfR and calnexin (CAL) in (**A**) DAT HEK cells or (**C**) G130T DAT HEK cells in the absence (-) or presence (+) of 100 mM ethanol. Total populations of transporter, TfR and calnexin were unchanged by ethanol in DAT and G130T DAT HEK cells. (**B** and **D**) Densitometric analysis of surface transporter and TfR immunoblots (surface levels were calculated as percent of total cellular DAT or TfR) expressed in (**B**) DAT HEK or (**D**) G130T DAT HEK cells (control, open bars; 100 mM ethanol, closed bars). Ethanol significantly increased surface expression of DAT by ~40% (*$p < 0.05$, unpaired t test; n=3) but had no significant effect on surface levels of G130T DAT (n=3). Ethanol had no effect on TfR levels in DAT or G130T DAT HEK cells. Values represent mean ± S.E.M.

4.3 Ethanol Action on DAT Endosomal Recycling Pool Size

To elucidate how ethanol modulates the cell surface DAT population, we first determined if ethanol increases DAT on the surface by altering the overall size of the endosomal recycling pool. For these experiments, we used a modified biotinylation assay described previously (Wang and Quick 2005). Biotin was applied to the cells in trafficking permissive temperatures (37°C). Cell surface proteins and proteins inserted in the plasma membrane were labeled by biotin during the course of the assay (see representative immunoblot, Figure 4.3.A). The biotinylated DAT fraction was compared to total cellular DAT to estimate the overall recycling pool size. Under basal conditions, 70-80% of the total DAT population was biotinylated after 30 min of trafficking and remained unchanged up to 90 min (data not shown). The size the DAT recycling pool was not significantly altered by 100 mM ethanol over the course of 60 min (70.2% ± 4.9 compared to 71.9% ± 9.2 of total DAT for vehicle and 100 mM ethanol exposed cells, respectively) (Figure 4.3.B)

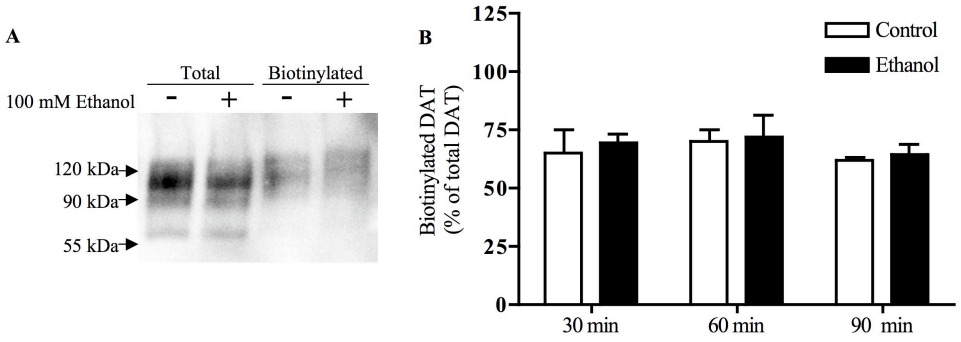

Figure 4.3: DAT recycling pool size is unchanged by ethanol. DAT HEK cells were exposed to 1 mg/ml biotin in the absence (-) or presence (+) of 100 mM ethanol for 60 min at 37°C (trafficking permissive). **A**. Representative immunoblot of total (Total) and recycling pool populations (Biotinylated) of DAT ± 100 mM ethanol. **B**. Densitometric analysis of DAT recycling pool immunoblots. Biotinylated transporter represents DAT within endosomal recycling pools (recycling pool size was calculated as biotinylated protein / total cellular DAT after 30,60, and 90 min of trafficking. Ethanol had no significant effect on DAT recycling pool size (unpaired t test, n=3). Values represent mean ± S.E.M.

4.4 Effects of Ethanol on DAT Cell Membrane Insertion Rate

DAT expression in the plasma membrane is regulated by a dynamic balance of transporter insertion in the cell membrane and endocytic internalization. We next asked if the ethanol-induced upregulation of cell surface DAT was due to changes in either one of these trafficking mechanisms. First, we determined if ethanol altered the rate of transporter insertion into the plasma membrane. For these experiments, we used modified biotinylation assays to measure DAT delivery to the cell surface over time. Assays were performed at 25°C to slow trafficking in order to accurately monitor ethanol action on transporter recycling. DAT HEK cells were first biotinylated under trafficking-

restrictive conditions (4°C) to label all surface transporters at 0 min (t =0). Cells were then incubated with biotin in trafficking permissive temperatures (25°C) for 0, 2, 5, 15 or 30 min (t) in the absence or presence of 100 mM ethanol. Biotinylated DAT was calibrated to the respective total cellular DAT for each time point. Newly inserted transporter levels were determined by comparing the percent increase in biotinylated DAT fractions at t = 2, 5, 15 or 30 min over DAT surface levels at t = 0 (see representative immunoblot, Figure 4.4.A). Data were fit to a single exponential function as described in Experimental Procedures (Figure 4.4.B), and estimations of basal and ethanol-modulated insertion rates were calculated (0.06 min^{-1} ± 0.01 and 0.24 min^{-1} ± 0.04 for control and 100 mM ethanol exposed cells, respectively). Both vehicle (control) and ethanol-exposed DAT HEK cells reached a plateau after 30 min where ~75% of total DAT was biotinylated. Ethanol significantly increased DAT insertion rate by ~4-fold. Immunoblots were re-probed for TfR (see representative immunoblot, Figure 4.4.C). Ethanol had no significant effect on TfR insertion rates in DAT HEK cells (0.22 min^{-1} ± 0.09 and 0.26 min^{-1} ± 0.12 for vehicle and 100 mM ethanol exposed cells, respectively) (Figure 4.4.D).

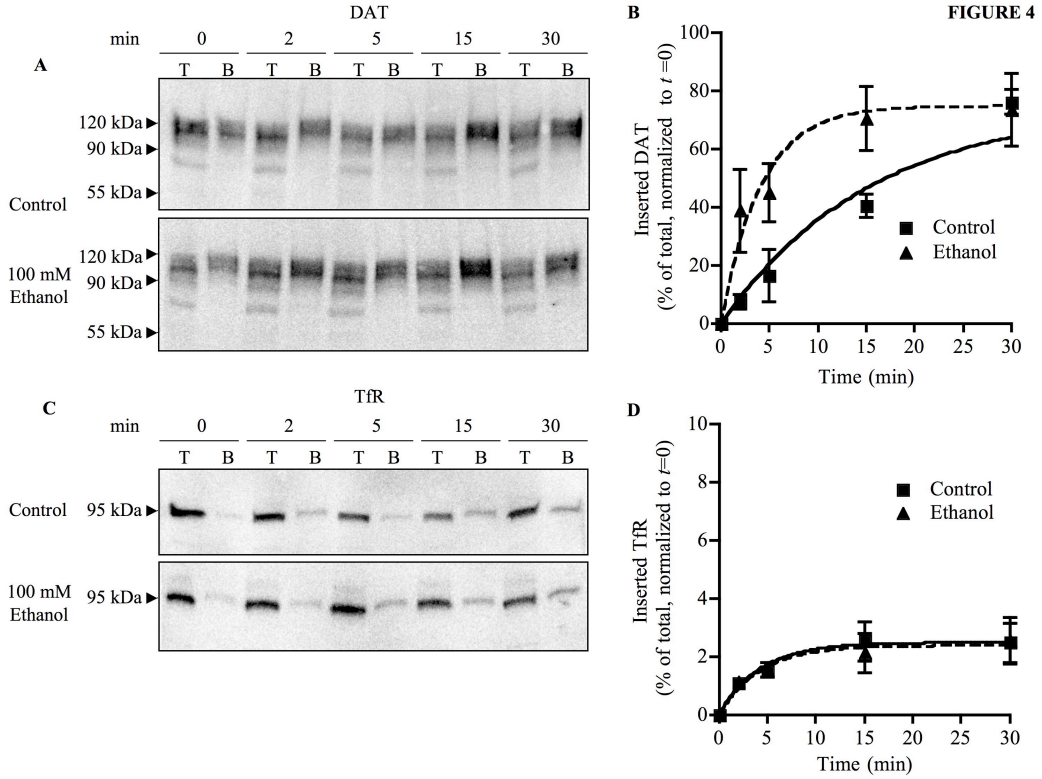

Figure 4.4: Ethanol increases DAT insertion rates. Transporter insertion into the membrane was observed using a modified biotinylation assay. (**A** and **C**) Representative immunoblots of total lysate (*T*) and biotinylated (*B*) populations of (**A**) DAT and (**C**) TfR in DAT HEK cells. Biotinylated samples represent newly inserted proteins into the cell surface at 25°C at 0, 2, 5, 15 and 30 min ± 100 mM ethanol. (**B** and **D**) Densitometric analysis of immunoblots plotted as a time course of (**B**) DAT and (**D**) TfR insertion into the cell surface of DAT HEK cells (control (■); 100 mM ethanol (▲)). Biotinylated proteins were calibrated to their respective total populations, and insertion was determined as the percent increase of biotinylated protein compared to basal surface levels at t = 0 min. Data were fit to a first order exponential equation as described in Experimental Procedures to calculate insertion rate. (**B**) Ethanol significantly increased DAT insertion rate compared to controls by ~ 4-fold ($p < 0.01$, F test). (**D**) Ethanol had no effect on TfR insertion into the surface of DAT HEK cells. Graphs represent the average of three experiments (± S.E.M.) at each time point.

4.5 Effects of Ethanol on DAT Internalization Rate

We next determined if ethanol altered endocytic internalization using reverse biotinylation assays. Transporters were labeled with biotin at trafficking-restrictive temperatures (4°C). Unbound biotin was removed, and cells were warmed to 25°C for 0, 2, 5, 15 or 30 min in the absence or presence of 100 mM ethanol. Cell surface-bound biotin was removed by an extracellular reducing agent. The remaining biotinylated DAT fraction represents internalized transporter. Internalized transporter was compared to the total surface (T.S.) DAT at *t*=0 (see representative immunoblot, Figure 4.5.A). Strip control samples indicate that 90-95% of biotin was removed during TCEP stripping washes (data not shown). Data was fit to a single exponential function as described in Experimental Procedures (Figure 4.5.B), and relative internalization rates were calculated (0.54 min^{-1} ± 0.09 and 0.6 min^{-1} ± 0.18 for control and 100 mM ethanol exposed cells, respectively). After 15 min, intracellular accumulation of transporter reached a plateau where 66.9% ± 2.6 or 38.13% ± 2.4 of total DAT was internalized in vehicle or ethanol-exposed cells, respectively. Ethanol had no significant effect on the rate of internalization. For TfR in DAT HEK cells (see representative immunoblot, Figure 4.5.C), ethanol had no significant effect on the total number of internalized receptors (38.2% ± 2.7 and 37.0% ± 2.8 of total TfR for vehicle and 100 mM ethanol-exposed cells, respectively) (Figure 4.5.D) or the internalization rate (0.18 min^{-1} ± 0.05 and 0.20 min^{-1} ± 0.08 for vehicle and 100 mM ethanol-exposed cells, respectively) (Figure 4.5.A and B).

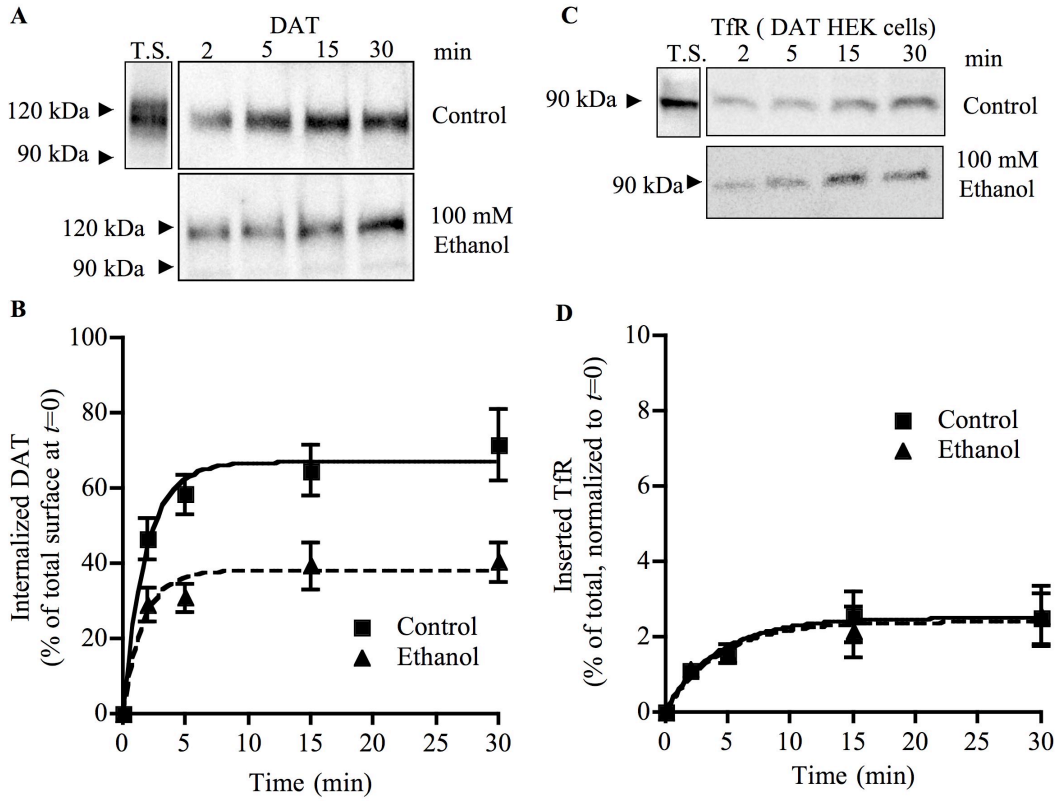

Figure 4.5: Ethanol has no effect on WT DAT internalization rates. Internalization of transporter was determined using reverse biotinylation assays. Intracellular accumulation of DAT and TfR in DAT HEK cells was measured at 2, 5, 15 and 30 min at 25°C ± 100 mM ethanol. (**A** and **C**) Representative immunoblots of basal total surface (T.S.) and internalized proteins in (**A**) DAT and (**C**) TfR in DAT HEK cells ± 100 mM ethanol. (**B** and **D**) Densitometric analysis of immunoblots plotted as a time course of (**B**) DAT and (**D**) TfR internalization from the cell surface of DAT HEK cells (control (■); 100 mM ethanol (▲)). Total number of internalized proteins was calculated as the percent of biotinylated protein at each time point compared to basal total surface levels (T.S.; t = 0). Data were fit to a first order exponential equation as described in Experimental Procedures to calculate internalization rate. (**B**) The total number of internalized DAT significantly decreased by ~50% after ethanol treatement ($p < 0.01$, F test comparison of time course maximums between control and ethanol treated cells) but produced no significant effect on internalization rate of the transporter. (**D**) Ethanol had no effect on the total number of internalized TfR or internalization rate. Graphs represent the average of three experiments (± S.E.M.) at each time point.

4.6 Discussion

Previous *in vitro* studies in DAT-expressing Xenopus oocytes and heterologous cell expression systems have shown ethanol potentiates [^{3}H]DA uptake (Mayfield et al. 2001, Riherd et al. 2008). Kinetic analysis of [^{3}H]DA uptake revealed these changes were not due to alterations in the binding affinity of the transporter for dopamine (Riherd et al. 2008). Ethanol-induced increases in transporter function were associated with an increase in [^{3}H]WIN 35,428 DAT binding and expression on the cell surface, suggesting ethanol may alter transporter trafficking (Mayfield et al. 2001, Riherd et al. 2008). However, the subcellular mechanisms underlying ethanol-mediated regulation of DAT surface expression are unknown. In this study, we found ethanol increases DAT-mediated [^{3}H]DA uptake by altering endosomal recycling of the transporter. Our data suggest ethanol increases the rate of insertion of DAT into the cell membrane, while having no effect on the rate internalization. These ethanol-induced modulations of the DAT endosomal trafficking pathway result in a net increase of functional transporters on the cell surface.

For these experiments, we used HEK-293 cells stably expressing DAT which were developed in a previous study (Riherd et al. 2008). The DAT HEK cells were utilized to directly examine the pharmacological action of ethanol on transporter function and trafficking. For comparison of function and surface localization, we developed another stable line of HEK-293 cells expressing an ethanol-insensitive mutant, G130T DAT. The G130T mutation was developed

from chimeric studies between DAT and NET (Maiya et al. 2002). Although closely related in structure and function, previous studies showed ethanol has opposing effects on DAT and NET (Lin et al. 1993, Lin et al. 1997, Maiya et al. 2002). G130T DAT HEK cells were used in this study as a tool to determine if DAT sensitivity to ethanol was mediated by specific alterations in surface expression of the transporter. Finally, we used a biochemical approach to investigate ethanol-induced changes on endosomal recycling of DAT. Examining ethanol modulation of trafficking *in vitro* allows us to determine the direct pharmacological actions of ethanol on intracellular trafficking of the transporter. A caveat to this approach is that we can only determine the relative rates and changes in intracellular trafficking. The use of cell lines, which over express the transporter, does not allow for an accurate measurement of recycling rates found in intact neurons. However, this experimental approach allowed us to determine ethanol-induced changes on the distinct steps involved in endosomal recycling and yielded novel insights into the mechanisms of ethanol action on subcellular trafficking of the transporter.

Characterization of DAT and G130T DAT function revealed the G130T mutation had no effect on basal [^{3}H]DA uptake; however, the point mutation abolished the ethanol sensitivity of transporter function. In contrast, previous observations with HEK-293 and neuronal SK-N-SH cell lines transiently expressing transporters showed that G130T reduced, but did not eliminate, ethanol sensitivity in these mammalian cell expression systems (Riherd et al.

2008). In these studies, stable expression of the transporters reduced variability in [^{3}H]DA uptake assays compared to cells used in our previous study, which transiently expressed the transporters. This reduction of variability between assays in the current study likely contribute to these differences in [^{3}H]DA uptake.

Analysis of ethanol-induced changes in DAT and G130T DAT surface expression indicated ethanol sensitivity of DAT was mediated by changes in surface expression and was specific for DAT. Ethanol induced a significant increase in cell surface DAT but had no effect on expression of G130T DAT in the membrane. Immunoblots revealed a population of the mutant transporter at ~65-70 kDa. This population likely contains immature transporter retained in the endoplasmic reticulum (ER). While the mutation does not interfere with known export, oligmerization or ER-retention motifs (Miranda et al. 2004), mutation of glycine 130 may alter folding of a portion of the transporter population, thus preventing export from the ER. However, a large portion of functional G130T transporter population is trafficked out of the ER. Ethanol had no effect on the surface populations of mutant transporter, and misfolding does not likely contribute to the lack of an ethanol effect on G130T DAT surface expression. The mutation also does not interfere with known residues critical in DAT surface retention (Sorkina et al. 2009). Therefore, ethanol-induced alteration of DAT surface expression does not appear to involve known signaling sequences required for constitutive trafficking of the transporter (Holton et al. 2005). Ethanol

also does not affect total cellular transporter levels or the size of DAT endosomal recycling pools. While not tested directly, these data suggest ethanol does not alter synthesis, secretion or lysosomal degradation of transporter. Endogenous TfR surface expression was also not altered by ethanol, confirming ethanol does not have a non-specific effect on endosomal trafficking. These results indicate the following: (i) ethanol-induced potentiation of DAT function is mediated by changes in surface expression of the transporter, (ii) this sensitivity is specific to DAT and (iii) ethanol-induced increase in surface expression is likely due to alterations in the endosomal recycling pathway and not overall expression changes.

Dynamic membrane trafficking of neurotransmitter transporters is essential for efficient regulation of the duration and strength of synaptic neurotransmission (Jayanthi and Ramamoorthy 2005). The balance between insertion and endocytic internalization of transporters within the recycling pathway maintains homeostatic transmitter levels. DAT and other neurotransmitter transporters recycle in a constitutive manner. However, intrinsic and pharmacological modulators can also regulate endosomal recycling, mediating changes in neurotransmission (Yu et al. 2000, Whitworth and Quick 2001b, Whitworth and Quick 2001a, Whitworth et al. 2002, Granas et al. 2003, Holton et al. 2005, Jayanthi and Ramamoorthy 2005, Maiya et al. 2006). Alterations in cell surface transporter expression arise from modulation of transporter insertion, internalization or both steps of the recycling pathway. For

example, amphetamine-regulated DAT trafficking results in a suppression of transporter insertion and stimulation of endocytic rates, causing an overall decrease in surface levels of the transporter (Boudanova et al. 2008a). Here we show that ethanol modulates distinct steps in the endosomal recycling of DAT. These novel findings suggest ethanol elevates surface expression of the transporter by increasing the rate of DAT insertion and without affecting the rate of internalization. The reduction of the overall number of transporters internalized by ethanol was likely a refection of depleted internal endosomal pools caused by the significant increase in insertion rate. Ethanol had no effect on TfR endosomal recycling, suggesting that TfR and DAT recycle in separate endosomal pools, and ethanol does not induce a generalized effect on trafficking of endosomes.

The molecular components involved in ethanol-induced regulation of DAT recycling are unknown. DAT does not contain classical trafficking signal sequence sites such as tyrosine- or dileucine-based motifs (Holton et al. 2005). Monoamine transporters are subject to regulation by PKC-dependent mechanisms; however, kinase-mediated phosphorylation is not required in regulated or constitutive trafficking of DAT (Chang et al. 2001). Furthermore, ethanol-sensitive areas of the transporter do not contain phosphorylation sites, suggesting ethanol-induced modulation of trafficking is not mediated through changes in the phosphorylation state of DAT. Accessory proteins are likely involved in ethanol modulation of exocytic insertion. Emerging evidence

suggests rapid regulation and clustering of other neurotransmitter transporters is dependent on interaction with microfilament proteins (Duan et al. 1999, Zhou and Sutherland 2004, Mochizuki et al. 2005, Imoukhuede et al. 2009). Ethanol has been found to reorganize the actin and microtubulin cytoskeleton involved in the glucose transporter trafficking, altering glucose uptake (Tomas et al. 2003). Further investigation is needed to define the trafficking proteome, and ethanol action on those protein-protein interactions (Torres 2006). In summary, the present study has revealed that ethanol modulates the DAT endosomal recycling pathway, thus directly altering transporter function. These novel findings are the first to describe ethanol effects on specific steps of the transporter trafficking pathway, and provide a framework to further understand the action of ethanol on DAT-mediated dopamine regulation.

Chapter Five

Characterization of the first intracellular loop of DAT in cell membrane trafficking

5.1 Introduction

The dynamic trafficking of DAT is critical in regulation of the transporter, and dopaminergic signaling. As outlined in chapter one, DAT trafficking occurs in a constitutive and regulated manner. The transporter recycles to the cell surface from intracellular endosomal pools to regulate DA levels in rapid and efficient manner. Multiple second messenger systems and various drugs of abuse can regulate transporter function by altering the cell surface distribution of the transporter. For example PKC activation and amphetamine cause an internalization of DAT in a clathrin- and dynamin-dependent manner (Zhang et al. 1997, Daniels and Amara 1999, Melikian and Buckley 1999, Saunders et al. 2000). Acute exposure of cocaine enhances DAT activity by altering intracellular trafficking of the transporter, increasing DAT cell surface expression (Daws et al. 2002, Little et al. 2002). Evidence presented in chapters three and four suggest ethanol also regulates DAT function by altering endosomal recycling of the transporter.

The molecular components and trafficking proteome involved in DAT trafficking remain unclear. The transporter does not contain classical trafficking signaling consensus sites (Holton et al. 2005), and phosphorylation of the transporter is not required for constitutive or regulated trafficking (Chang et al. 2001). Trafficking of DAT could be modulated by changes in protein-protein interactions. Intracellular loops are accessible to interaction with accessory proteins, and modifying enzymes (Quick et al. 1997). Ethanol-induced regulation of the transporter is mediated by critical amino acids in the first intracellular loop, none of which are contained in phosphorylation consensus sites (Maiya et al. 2002, Riherd et al. 2008). Studies in chapter four suggest mutation of G130 in IL1 alters DAT sensitivity to ethanol by inhibiting the trafficking-dependent upregulation of DAT surface localization. Chimera studies between DAT and NET, which is inhibited by ethanol, in Xenopus oocytes suggest this loop is critical in DAT trafficking and ethanol-mediated regulation of transporter trafficking.

NET and DAT share a high degree of homology in amino acid sequence and function. The two transporters differ by four amino acids in IL1, DAT residues F123, G130, I137, and L138. In Xenopus oocytes, individual point mutations of G130 and I137 reduced ethanol sensitivity of the transporter, while F123 and L138, demonstrated no significant ethanol-induced changes (Maiya et al. 2002). Mutation of all four sites to the homologous NET residues resulted in a complete abolishment of [^{3}H]DA uptake and [^{3}H]WIN 35,428 surface binding of DAT in

oocytes. These results suggest IL1 is important in constitutive and ethanol-regulated trafficking of the transporter to the cell surface.

The objective of aim three is to characterize the function and cellular localization of the IGLF DAT in mammalian cells. The generation and characterization of the IGLF mutant may be important in future studies identifying protein interactions critical in DAT trafficking. We hypothesize IGLF DAT uptake function will be abolished as a result of deficiencies in cell membrane trafficking.

5.2 Functional Characterization of IGLF DAT HEK

Stably expressing IGLF DAT HEK cells containing an eGFP tag were generated, as described in chapter two. Clonal colonies were selected, and expanded based on IGLF DAT expression monitored by fluorescence microscopy. Colonies with 90-100% of the cells expressing the mutant were selected for expansion, and expression was verified with immunoblotting (Figure 5.1.A). The function of IGLF DAT was examined using [^{3}H]DA uptake assays. Stably expressing DAT or IGLF DAT cells were incubated with 20 nM [^{3}H]DA for 3 min, and dopamine uptake was measured as the accumulation of [^{3}H]DA into the cells. Compared to DAT HEK cells (0.15 ± 0.02 pM/min/ 0.05x10^5 cells) the IGLF DAT cells exhibited a complete abolishment of [^{3}H]DA uptake function (-0.0003 ± 0.0002 pM/min/ 0.05x10^5 cells) (Figure 5.1.B).

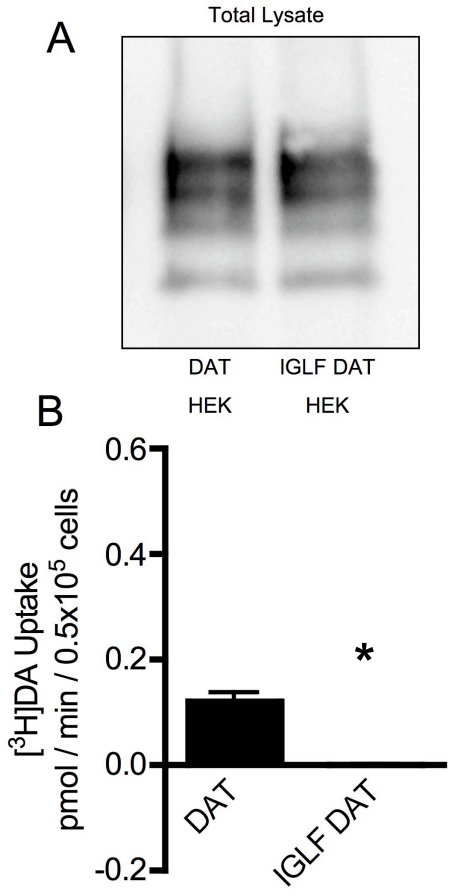

Figure 5.1: IGLF mutation of DAT abolishes transporter function. A. DAT or IGLF DAT was stably expressed in HEK-293 cells. Expression was verified by immunoblotting with DAT antibody (MAB 369). Representative immunoblot of ~80μg total lysate. B. Basal transporter-mediated [^{3}H]DA uptake. The IGLF mutation abolished transporter function (*p < 0.01 compared to DAT HEK; unpaired t test; n= 7 assays). Values represent mean ± S.E.M

5.3 Cell Surface Expression Analysis of IGLF DAT

Cell surface expression of IGLF DAT was measured to determine if the inhibition of [^{3}H]DA DAT-mediated uptake was due to a lack of transporters on the cell surface. Biotinylation assays, a direct measure of cell surface protein expression, were used to compare surface localization of DAT and IGLF DAT. The cell surface population of proteins was measured as a percentage of the total amount of the respective cellular populations (Figure 5.2). Surface DAT levels (36.6% ± 8.5 of total DAT population) were not significantly different from surface IGLF DAT levels (24.4% ± 4.2 of total IGLF DAT populations). Blots

were stripped and reprobed with a TfR antibody, an endogenous protein which undergoes endosomal recycling. Cells stably expressing the IGLF mutant also had no significant difference in TfR levels compared DAT HEK cells (8.6% ± 0.4 compared to13.3% ± 7.0 TfR surface expression in DAT and IGLF DAT cells, respectively).

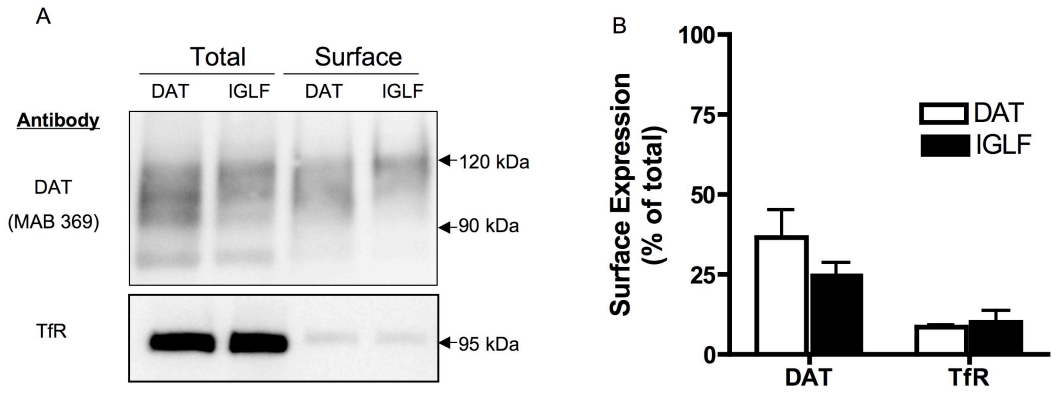

Figure 5.2: The IGLF DAT mutation has no effect on surface localization. **A**. Representative immunoblot of total (Total) and biotinylated (Surface) populations of transporter and TfR in DAT HEK cells (DAT) or IGLF DAT HEK cells (IGLF). **B.** Desitometric analysis of surface transporter and TfR immunoblots. Surface expression was calculated as perecent of total cellular DAT or TfR expressed in DAT HEK or IGLF DAT HEK cells. Total and surface populations of transporter (DAT and IGLF) and TfR were unaffected by the IGLF mutation (unpaired *t* test; n=3). Values represent mean ± S.E.M.

5.4 Discussion

The objective of aim three was to characterize IGLF DAT function and surface localization in mammalian cell systems in order to potentially use the mutant as a tool in delineating the molecular mechanisms of transporter trafficking. IGLF DAT function was completely abolished, however surface levels of the mutant were not significantly different from cells expressing wild-type

transporter (DAT HEK). These results were unexpected, and differ from the findings from Xenopus oocytes (Maiya et al. 2002), which suggested the inhibition of the mutant transporter function was due to an impairment of trafficking to the cell surface.

Discrepancies between the transporter cell surface localization results from this study and those found using the Xenopus expression system are likely due to differences in the methods used to measure surface expression. In the Xenopus oocyte expression system, surface localization of DAT was measured using [^{3}H]WIN 35,428 binding. [^{3}H]WIN 35,428 is a potent cocaine analog, and binds to the cocaine-binding site of the transporter, which overlaps the DA binding site. In this study, surface expression was measured directly using a biochemical labeling method, biotinylation. Both the mammalian cell and Xenopus expression systems expressing IGLF DAT demonstrated a lack of function. These results suggest IGLF mutant DAT cannot bind or translocate DA, which is likely the reason [^{3}H]WIN 35,428 binding could not detect transporter on the cell surface.

The IL1 of DAT is not required for constitutive trafficking of the transporter to the cell surface as previously speculated (Maiya et al. 2002), but may play a role in the conformational requirements of DAT for DA binding. This hypothesis is supported by analysis of the $LeuT_{Aa}$ crystal structure, which suggests TM3 and TM8 shift during DA binding and translocation into an inward-facing position, expanding IL1 and allowing DA permeation (Yamashita et al. 2005). A crystal

structure depicting an unbound transporter is not available, but the analysis of the role of IL1 in the translocation of substrate in $LeuT_{Aa}$ structure suggests the mutations of IGLF DAT may cause DAT to be maintained in the inward-facing position, not allowing for DA binding. Interestingly, a study examining the role of DAT TM2 in cocaine binding found mutation of Q122, the residue immediately adjacent to F123 mutated in IGLF DAT, also resulted in a transporter which lacked function which was expressed on the cell surface (Sen et al. 2005). The researchers suggested the cytoplasmic face of TM2/IL1 (location of Q122 and F123) region interacts with TM6, one of the regions directly involved in substrate binding. The mutations likely perturb the interaction of TM2-TM6, causing the transporter to be maintained in the inward-facing position, and indirectly inhibiting ligand binding. Further investigation of the IGLF DAT-induced conformational changes, and its role in DA binding is needed to confirm this hypothesis.

Chapter Six: Discussion

The midbrain dopaminergic system, which includes DA neurons projecting to the striatum, NAc, and the PFC from the SN and VTA, plays a central role in mediating the reward and reinforcing effects of drugs of abuse (Hyman et al. 2006). These drugs, including psychostimulants and ethanol, elevate synaptic DA levels, mediating reinforcement. Proper regulation of DA neurotransmission maintains homeostatic levels of the transmitter in active synaptic zones, and is mediated by a reuptake mechanism by the monoamine transporter, DAT. Localized on the peri-synaptic areas of pre-synaptic DA terminals, DAT is the primary site of action of several psychostimulants, including cocaine and amphetamine. Psychostimulants elevate DA levels by blocking DAT-mediated DA reuptake, and ethanol increases the transmitter level by enhancing DA release (Gainetdinov and Caron 2003, Gonzales et al. 2004). Unlike other drugs of abuse, ethanol is known to alter the function of multiple neurotransmitter systems and proteins in many brain regions. The findings presented from the present studies outline the potential role of ethanol in altering the function and regulation of DAT.

6.1 Ethanol Action on DAT Function in Mammalian Systems

Ethanol alters various properties of the dopaminergic system, including enhancing synaptic DA concentrations by increasing release of the transmitter

(Imperato and Di Chiara 1986, Di Chiara and Imperato 1988, Weiss et al. 1993, Yim and Gonzales 2000). Few studies have investigated the role of transporters on ethanol-induced changes in synaptic transmission. The effects of ethanol on DAT-mediated DA regulation are controversial. Our studies suggest that short-term ethanol exposure potentiates DAT function in a concentration-dependent manner when expressed in mammalian cells. These results are in agreement with previous findings in Xenopus oocytes expressing the transporter. However, *in vivo* and *ex vivo* electrochemical studies, including results from microdialysis, voltametry, and chronoamperometry, suggest ethanol may increase, decrease or have no effect on DA clearance (Wang et al. 1997, Budygin et al. 2001, Sabeti et al. 2003, Robinson et al. 2005, Mathews et al. 2006).

The varied findings from electrochemical experiments are likely a reflection of the differences in experimental methods, electrical stimulation, mechanisms of ethanol administration, and ethanol concentration. For example, researchers using the quantitative no-net-flux microdialysis technique have suggested ethanol-induced DA increases in rat NAc are not due to an inhibition of DAT activity (Yim and Gonzales 2000). The investigators also found dialysate DA levels return to baseline while extracellular ethanol concentrations remain high. The temporal difference in dialysate DA and ethanol levels could result from increased transporter function (Mayfield et al. 2001), however, this technique provides only an indirect measurement of transporter activity, and cannot measure potential increases in uptake (Yim and Gonzales 2000, Tang et

al. 2003a). The microdialysis technique also has a low temporal resolution with dialysate sampling taking 5 to 20 min per a sample, potentially missing transient drug-induced changes in DA clearance.

Voltammetry allows for a better temporal resolution, however the *in vivo* prep requires animals to be anesthetized during the experiment. There are relatively few studies investigating the effects of anesthesia on DAT. However, it has been reported that chloral hydrate, propofol, ketamine, halothane and isoflurane alter DAT uptake kinetics and/or cell surface expression (Shahani et al. 2002, Sabeti et al. 2003, Byas-Smith et al. 2004), suggesting anesthesia may alter results from voltammetry studies of DAT-mediated DA uptake.

Measurements of DAT activity in mammalian cell expression systems allow for a direct assessment of transporter activity and potential correlation with changes in DAT regulation. However, this *in vitro* approach may lack potentially vital cellular connections and cell- tissue-specific signaling needed for a gross assessment of the drug on an organism level. The *in vitro* model is beneficial in analyzing the pharmacological drug action on specific proteins. *In vitro* investigations of ethanol action on DAT expressed in neuronal and non-neuronal mammalian expression systems, have suggested ethanol enhances transporter function only in neurons (Ho and Segre 2001). However, we found ethanol potentiates DAT when expressed in neuronal or non-neronal cell lines, suggesting ethanol does not require a neuronal cell-specific pathway. Differences between these studies could be attributed to cell type and/or transfection

efficiencies. The study conducted by Ho and Segre (2001) compared CHO cells to Neuro2A neuroblastoma cells to examine neuronal cell-specific effects of the transporters. Others have found this cell line is particularly sensitive to passage number when transiently expressing protein, including DAT, resulting in changes of basal DA uptake kinetics, and possible discrepancies in drug-induced changes of function (Ukairo et al. 2007).

When we transiently expressed DAT into SK-N-SH or HEK-293 cells, ethanol-induced potentiation of function was variable and lacked an overall concentration-dependent pattern of uptake. The G130T DAT transient expression resulted in a significant, but lower, potentiation of uptake. These differences could be attributed to 1) variability of transient transfection expression, 2) low level of endogenous DAT in SK-N-SH cells, 3) leaky membranes from transfection methods, or 4) lack of an ethanol effect in mammalian systems. In contrast, stable expression of the transporter revealed a concentration-dependent effect of ethanol (chapter four), suggesting the variability of uptake between assays in the earlier study (chapter three) was a result of variability of transient expression. Furthermore, a point mutation of one amino acid in IL1 of DAT (G130), alters this sensitivity, also suggesting ethanol modulates DAT in a direct manner. Overall, our findings in mammalian cells are consistent with previous studies in a *Xenopu*s expression system system showing ethanol potentiates DAT-mediated DA uptake.

6.2 Ethanol-Mediated Alterations of DAT Regulation

Ethanol-induced enhancement of DAT-mediated uptake correlated with an increase in cell surface expression of the transporter. The G130T DAT mutant was insensitive to ethanol, and surface expression of the mutant did not change after ethanol exposure. These findings further suggest ethanol-induced changes of transporter function are trafficking mediated. The focus of the second aim was to determine the mechanism involved in mediating redistribution of DAT to the cell surface. Short-term ethanol exposure could enhance cell surface expression of the transporter by several mechanisms 1) increasing the number of DAT in the endosomal recycling pool 2) increasing the rate of insertion 3) decreasing the rate of internalization or 4) a combined effect of increasing insertion and decreasing internalization.

For these studies we assessed cell surface trafficking rates of the transporter using modified and reverse biotinylation assays. The advantage to the biotinylation method is that it allows us to directly assess recycling of transporters over time, and monitor insertion and internalization separately. The use of these assays in measuring transporter trafficking is relatively new (Wang and Quick 2005) and has never been used to detect drug-induced action on multiple steps of the DAT trafficking pathway. Caveats to this approach are that we cannot directly compare rates of insertion to rates of internalization due to differences in the experimental methods, and the use of cell lines, which over

express the transporter, does not allow for an accurate measurement of recycling rates as found in intact neurons. However, this experimental approach allowed us to pinpoint ethanol-induced changes on the distinct steps involved in endosomal recycling and yielded novel insights into the mechanisms of ethanol action on subcellular trafficking of the transporter.

Due to the ubiquitous effects of ethanol, we considered that ethanol might affect multiple steps of the endosomal recycling pathway. However, we found ethanol only modulated insertion of the transporter (Figure 6.1). Furthermore, ethanol had no effect on endogenous proteins that undergo similar endosomal recycling or the size of the DAT endosomal recycling pool. These results suggest ethanol effects were specific to the transporter, and did not alter the machinery involved in endocytic internalization of DAT.

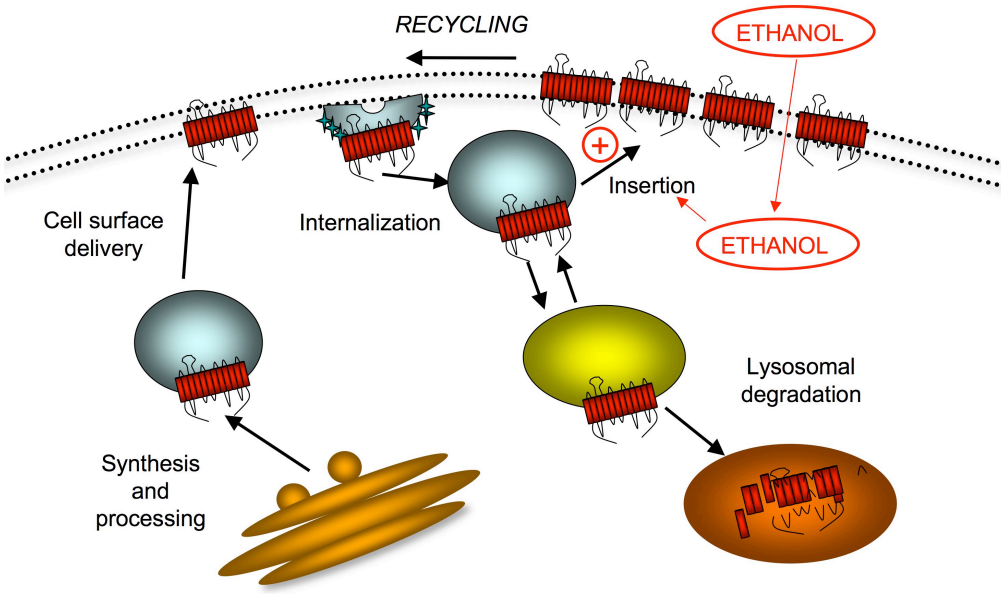

Figure 6.1: Ethanol action on DAT endosomal recycling. Ethanol potentiates DAT function by increasing the rate of insertion, causing an enhancement of cell surface localization of DAT. Internalization and DAT recycling pool size are not altered by ethanol.

The mechanism of ethanol-induced changes on the exocytic insertion of DAT remains unclear and could involve several molecular components the DAT trafficking proteome. Future analysis of DAT, compared to the ethanol-insensitive G130T DAT, could yield insight into potential protein interactions disrupted by ethanol. DAT does not contain classical trafficking signal motifs, and its non-classical signal motif, which is conserved among all monoamine transporters, was identified in the C-terminal region of the transporter. We found the IL1 was important in mediating ethanol sensitivity; therefore, ethanol does not appear to interfere with interactions with this motif.

The G130T mutation does not interfere with known phosphorylation sites. However, potential ethanol-mediated changes in kinase activity could alter transporter trafficking indirectly via other kinase-stimulated protein interactions. Interestingly, one difference between NET and DAT is the effect of PKA pathway activation. Stimulation of the adenylyl cyclase-cyclic AMP (cAMP)-PKA pathway by forskolin or cAMP analogues potentiates DA uptake in rat striatal synaptosomes (Batchelor and Schenk 1998, Page et al. 2000), however, forskolin stimulation inhibits the function of NET expressed in PC12 cells (Nakanishi et al. 1995). Furthermore, acute ethanol treatment has been shown to modulate several aspects of the PKA pathway in mouse brain, including PKA-dependent phosphorylation of trafficking-related proteins (Hoffman and Tabakoff 1990, Conti et al. 2009). These findings open the possibility of a PKA-stimulated protein altering DAT trafficking through its interactions at IL1.

Ethanol could also modulate the interaction of microfilaments with the transporter. Dynamic modulation of the cytoskeleton, which is primarily composed of actin and microtubules, is critical for proper protein trafficking. The activity of Eps8, a protein that regulates actin dynamics, was found to be sensitive to ethanol (Offenhauser et al. 2006). Acute ethanol treatment induced actin reorganization in an Eps8-dependent manner, altering NMDA receptor trafficking. Ethanol has also been shown to disrupt microtubules, altering synaptic trafficking in rat brain (Ahluwalia et al. 2000). However, an ethanol-induced reorganization of the cytoskeleton would likely result in more global changes on protein trafficking, which we did not observe.

DAT, as well as other monoamine transporters, have been shown to traffic and function as homo-oligomers (Sorkina et al. 2003, Torres et al. 2003a). Recently, studies investigating the relationship between DAT oligomerization and pharmacological-regulated trafficking by amphetamine and cocaine have suggested the substrates can modify oligomerization (Chen and Reith 2008). In addition to reducing surface DAT levels, amphetamine also reduces DAT-DAT interactions. Blockade of amphetamine-induced endocytosis counteracted the reduction of oligomerization by the drug. Interestingly, cocaine, which increases DAT surface expression, increased DAT oligomerization. These important findings are the first to show a pharmacological regulation of oligomerization and the potential linkage to transporter trafficking. It would be interesting to

investigate whether ethanol-induced regulation of endosomal recycling is associated with post-translational changes in oligomerization.

Recent investigations of the involvement of cell surface interfaces of TM2-IL1 in oligomerization have ruled out the region in DAT-DAT interactions, but suggest a role in DA bindings (Sen et al. 2005). Our findings with the IGLF mutant DAT support these findings. The multiple mutations of could also just be causing a misfolding of the protein, rendering the transporter dysfunctional. However, IGLF DAT traffics to the cell surface similarly to WT DAT. While these results force us to reject our hypothesis that the IL1 is required for constitutive trafficking, it would be informative to investigate the role of the region in conformational changes required for substrate binding.

6.3 Implications of Ethanol Action on DAT

Ethanol-mediated enhancement of DAT function likely reflects a compensatory mechanism to counter-balance elevated synaptic DA concentrations induced by ethanol. These changes could contribute to an acute functional tolerance observed for DA-mediated responses (Fitzgerald and Nestler 1995). The ethanol-enhanced function and alteration of endosomal recycling of DAT could, in particular, negatively impact individuals with abnormal transporter levels. For example, studies have suggested ethanol-modulated DAT levels could contribute to the severity of acute withdrawal symptoms. A recent study suggested that alcoholics undergoing acute withdrawal actually have elevated levels of the transporters, contributing to the severity of the withdrawal symptoms

(Cosgrove et al. 2009). The elevated transporter levels could lead to a decrease DA tone, and a greater vulnerability to alcohol dependence. Acute ethanol exposure in these patients could further enhance surface DAT levels, leading to an acute tolerance effect, and a possible increase in drinking.

The findings from the studies presented here describe ethanol effects on DAT function and specific steps of the transporter trafficking pathway. Ethanol potentiates DAT-mediated DA uptake by altering regulation of the transporter. We found ethanol specifically increases the rate of DAT insertion, resulting in a redistribution of the transporter onto the cell surface. These novel findings are the first to describe a molecular mechanism of ethanol action on DAT, and provide a framework to further understand the action of ethanol on synaptic dopamine regulation.

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